The Dissertation Committee for Barry D Davis certifies that this is the approved version of the following dissertation:

Structural Characterization of Isomeric Flavonoid Glycosides and Metabolites by Metal Complexation and Electrospray Ionization Tandem Mass Spectrometry

Committee:

_____
Jennifer S. Brodbelt, Supervisor

_____
James A. Holcombe

_____
Michelle A. Lane

_____
Lara K. Mahal

_____
Jason B. Shear

Structural Characterization of Isomeric Flavonoid Glycosides and Metabolites by Metal Complexation and Electrospray Ionization Tandem Mass Spectrometry

by

Barry D Davis, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

the University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December 2007

Acknowledgements

The work presented in the following pages was assisted by numerous people. My advisor, Dr. Jennifer Brodbelt, is thanked for providing me with just the right balance of supervision and independence. I was free to make mistakes and learn from them while always having access to her wealth of knowledge. Under her tutelage, I have grown immensely as a scientist. Many thanks also go to the members of the Brodbelt research group for their advice, encouragement, and limitless supply of camaraderie and diversion during the tough times.

My scientific collaborators deserve special thanks for their contributions, as many of the applications of this work would not have materialized without them. Jeffrey Williams, Małgorzata Wojcińska and Prof. Tom Mabry approached me with the *Silphium albiflorum* project, which provided the first opportunity to use the described methods to produce useful data that could not be easily obtained in any other way. With that project, my research crossed the line from being merely interesting, to being useful and effective in practical applications. Paul Needs and Paul Kroon donated the initial training set of synthetic flavonoid glucuronides which made all of the subsequent metabolites work possible. They also provided samples from human intervention and cell culture studies. Our collaboration was definitely a fruitful one (this time pun intended). Prof. Michelle Lane, Erik Wilder and Kally O'Reilly are thanked for invaluable advice and assistance in performing enzymatic syntheses of flavonoid metabolites.

Finally, I thank all those family members who have always believed in me. This work is dedicated to you.

Structural Characterization of Isomeric Flavonoid Glycosides and Metabolites by Metal Complexation and Electrospray Ionization Tandem Mass Spectrometry

Publication No. _____

Barry D Davis, Ph.D.

The University of Texas at Austin, 2007

Supervisor: Jennifer S. Brodbelt

Flavonoids form a vast group of natural products that occur ubiquitously throughout the plant kingdom. These compounds play a significant role in the field of phytochemistry and are of nutritional interest due to numerous reports of their benefits to human health. Structural characterization of individual flavonoid derivatives is challenging because of widespread isomerism and a lack of sensitive and specific analytical techniques. The goal of this work is to present practical tandem mass spectrometry methods for systematic isomer differentiation of flavonoid glycosides and flavonoid glucuronides.

Metal complexation is used extensively as a strategy to achieve this aim. In this approach, flavonoid derivatives and metal ions are mixed in solution,

resulting in the rapid self-assembly of complexes which are subsequently infused into a quadrupole ion trap mass spectrometer. Collision-induced dissociation of these flavonoid/metal complexes results in fragment ions that are highly characteristic of specific structural features of the flavonoid derivatives. These methods are adaptable to LC-MS analysis via post-column addition of the complexation reagents.

Methods to differentiate the five most common glycosylation sites of monoglucosyl flavonoids are described. Based on the fragment ions yielded from magnesium or manganese complexes, specific indicators of 3-O-glucosylation, 7-O-glucosylation, 4′-O-glucosylation, 6-C-glucosylation and 8-C-glucosylation are observed. The manganese complexation method also differentiates isomeric glucose and galactose sugars at the 3 position, as well as arabinose and xylose sugars. Differentiation of isomeric flavonoid glucuronide metabolites is achieved by cobalt complexation with auxiliary ligands.

The effectiveness of these methods is proven in numerous practical applications. Flavonoid glycosides are identified in extracts from apples, onions, and *Silphium albiflorum*. Flavonoid glucuronide metabolites are identified in urine samples and a cell culture extract. The identifications are achieved without the use of standards or additional analytical techniques. Finally, an enzymatic synthesis of flavonoid glucuronides is used to establish the regioselectivity of UGT1A1, an enzyme involved in flavonoid metabolism. The cobalt

complexation method successfully identifies many hitherto uncharacterized metabolites. The significance of this work lies in its potential application to problems in botanical, agricultural, nutritional and disease-prevention studies in which precise flavonoid identification is required.

Table of Contents

Chapter 1: Introduction

1.1 FLAVONOIDS: OCCURRENCE AND SIGNIFICANCE

Flavonoids form a vast group of natural products comprising thousands of individual members. *The Handbook of Natural Flavonoids*[1] lists 6467 known naturally-occurring members of the flavonoid family, a number that has surely grown since the handbook's compilation in 1999. Flavonoids are found universally throughout higher plants, as well as in many lower plants such as mosses and liverworts.[1] Plants use them for various purposes, including protection from solar radiation in the ultraviolet wavelengths, internal regulators, chemical messengers, and defense against insect herbavores.[2] Some flavonoids (the anthocyanidins) are very brightly colored, and are responsible for most red, blue and violet colors of fruit and flowers.[2] Although flavonoids are ubiquitous throughout the plant kingdom, each plant species tends to produce a characteristic array of flavonoids, with closely-related species producing similar compounds. For example, 8-hydroxy derivatives of the flavonoids apigenin and luteolin are found mainly in the Lamiaceae (mint) family.[2] The correlation between genetic relationships and flavonoid occurrence has made flavonoid analysis an important tool in the study of plant chemotaxonomy (the classification of species based on chemical composition).

As flavonoids are present in nearly all plant tissue, they make up an integral part of the human diet. However, there is disagreement over the amount of dietary exposure to these compounds. A 1976 report by Kuhnau estimated the mean flavonoid intake of Americans at approximately 1 g/day,[3] but this value has since been judged far too high. More recent studies done in 1995[4] and 2002[5] estimated American flavonoid intake at 12.9 mg/day and 20-22 mg/day, respectively. But a 2007 study revised these values up to 189.7 mg/day.[6] The inconsistency of these estimates can be attributed to different subsets of flavonoids quantified, different quantification methods, and a lack of thorough knowledge regarding the flavonoid content of different food items. These problems underscore the importance of developing comprehensive and reliable methods for analyzing the flavonoid content of foodstuffs.

Flavonoids have generated much interest from nutritionists and biochemists in the past decade or so due to their purported role in disease prevention, particularly of cardiovascular disease and cancer.[7-19] However, the evidence for such health benefits is inconsistent and controversial. In 2005, Arts and Hollman conducted a meta-analysis of published epidemiological studies tracking flavonoid intake and incidence of disease.[20] Seven[12,21-26] out of twelve[12,21-31] cohort studies for coronary artery disease reported a correlation between flavonoid intake and reduced incidence of disease; however one of these studies showed significant risk reduction only for men, not for women.[22] Another

study reported significant effects only in men with previous incidence of coronary artery disease.[29] Conversely, one study reported a *positive* correlation between flavonoid intake and coronary heart disease.[30] Only two[31,32] out of five[23,24,31-33] epidemiological studies found a decreased risk of stroke due to flavonoid intake. Studies dealing with flavonoids and cancer show some evidence of reduced risk of lung[34,35] and colorectal cancer[35] attributed to dietary flavonoids, but no reduced risk of stomach, urinary tract, prostate, breast, testicular or ovarian cancers.[20]

In addition to these epidemiological studies, a large number of *in vitro* experiments have provided evidence of the beneficial effects of flavonoids on human health. Yet these findings are also disputed, as *in vitro* studies frequently employ conditions that are not realistically achieved *in vivo*. For example, an informal survey of twelve *in vitro* studies[36-47] on the effects of flavonoids on cancer cells published in peer-reviewed journals during the first five months of 2007 reveals some disturbing trends. Of these twelve studies, ten report anti-cancer effects such as apoptosis or decreased cell proliferation at flavonoids concentrations exceeding 10 μM. However, peak plasma concentrations occurring in humans after consuming flavonoid-rich meals are typically around 1 μM at best.[48-52] Only one[36] of the twelve studies reports any activity at such low concentrations, and many of the studies do not even test the effects of exposure at these concentrations. Even worse, all twelve of the studies treat the cancer cells with the aglycon forms of various flavonoids, but most flavonoids (except

catechins) that are absorbed by the human body undergo rapid metabolism such that the chemical structures living cells are exposed to are different from the ones consumed in food.[50] These metabolites usually demonstrate lower bioactivity than the original molecules.[53] Not one of the twelve studies employs flavonoids in the relevant biochemical form. For these reasons, the value of *in vitro* studies of flavonoids has recently been called into question.[54,55] It has been argued that in order to gain true insight into the health benefits of flavonoids, it is the metabolic forms that must be studied, not the precursors that occur in food items. However, knowledge about these metabolites remains limited and very few have been positively identified.[48] There is an urgent need for new analytical tools and procedures for probing these compounds.

1.2 CHEMICAL STRUCTURE AND NAMING CONVENTIONS OF FLAVONOIDS

The basic chemical structure of all flavonoids consists of two aromatic six-carbon rings linked by a three-carbon bridge, in a C_6-C_3-C_6 motif. The characteristics of the three-carbon bridge determine the class of flavonoid, while substituents on the two C_6 rings distinguish individual members of each flavonoid class. Some of the possible variants of the bridge include cyclization, hydroxylation, oxygenation, charging, and double-bonding. As a result of all these variants, there are over a dozen classes of flavonoids. Prototypical structures of several of these classes are shown in Figure 1.1. Flavonols, flavones

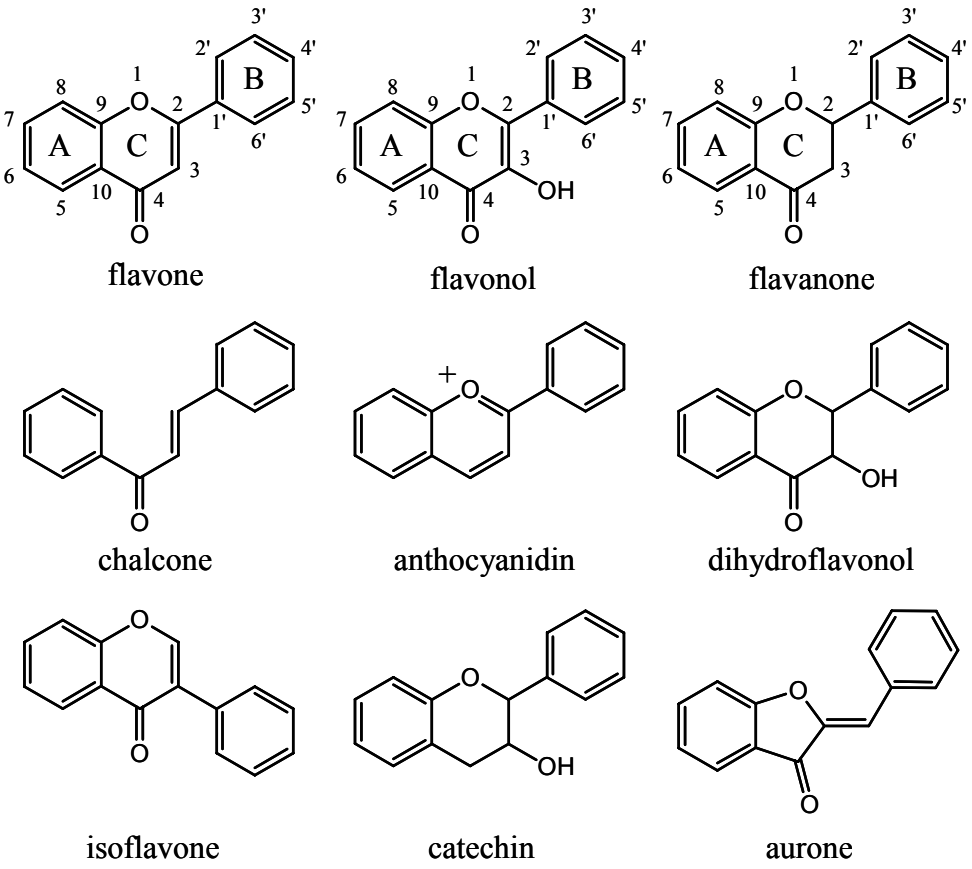


Figure 1.1: Prototype structures of several classes of flavonoids. Traditional numbering scheme and ring names are indicated.

and anthocyanidins are distributed throughout the plant kingdom, while the remaining classes are far less widely-occurring. For example, isoflavones are limited to the Faboideae, one of three subfamilies of Fabaceae (legumes) that includes soybeans and related plants. Flavanones are highly characteristic of *Citrus* and related genera. Catechins are found in a few specific plants such as

cocoa and tea.[1] In this dissertation, the focus will be on three of these classes: flavones, flavonols and flavanones.

The simplest flavonoid structures are aglycons, those compounds lacking attached saccharide moieties. The aglycons that comprise each flavonoid class differ in the number and locations of hydroxyl and methoxyl groups distributed around the flavonoid skeleton. The structures of all flavonoid aglycons mentioned in the following chapters are given in Figure 1.2. Flavonoid aglycons may be referred to by either trivial or systematic names, but the trivial names are often preferred for their simplicity (e.g. luteolin vs. 5,7,3′,4′-tetrahydroxyflavone) Throughout this dissertation, aglycons and the aglycon portion of flavonoid derivatives will be referred to only by their trivial names.

Flavonoids in plants are typically encountered as glycosides, meaning that one or more saccharide moieties have been attached to the flavonoid aglycon. These saccharides are most often attached via hydroxyl group oxygen atoms, forming compounds that are collectively known as flavonoid O-glycosides. While any hydroxyl group may be the site of O-glycosylation, some naturally occur far more often than others. In flavonols the 3-hyrdoxyl group is the most common glycosylation site, while the 7-hydroxyl group is most common for flavones and flavanones.[56] A saccharide group attached directly to a flavonoid carbon atom (forming a C-glycosyl flavonoid) may also occur, but this situation is limited to members of the flavone class, and even then C-glycosylation is only

known to occur at carbons 6 and 8 on the flavone skeleton.[56-58] Several different and often isomeric monosaccharides have been found in naturally-occurring flavonoid glycosides. Glucose is the most common, but galactose, rhamnose,

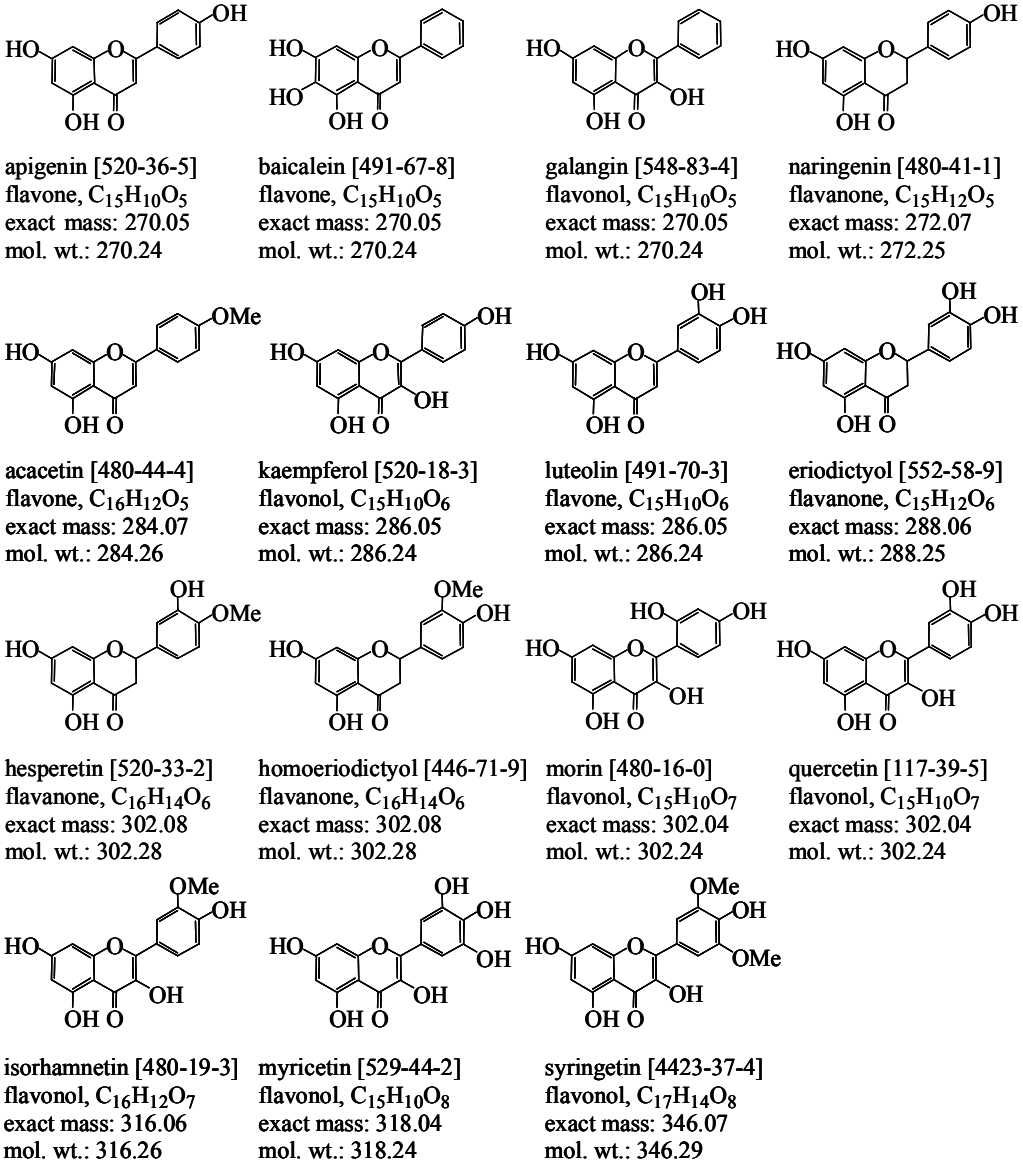


Figure 1.2: Chemical structures of flavonoid aglycons analyzed or mentioned in this work. CAS numbers are given in brackets. Class, chemical formula and mass are also provided.

arabinose and xylose are often observed as well. Mannose, fructose, allose, apiose, glucuronic acid and galacturonic acid are also encountered in plant flavonoids, but more rarely.[56]

The presence of saccharides greatly complicates the structural analysis of flavonoids. To properly identify a flavonoid glycoside, it is necessary to identify not only the aglycon and all of the attached saccharides, but also the position of attachment of each saccharide moiety. To further complicate matters, most glycosyl groups can occur in either a five-membered ring (furanose) or a six-membered ring (pyranose) form. In practice, however, only arabinose exhibits this variability as far as flavonoid glycosides are concerned; the other commonly-occurring saccharide groups nearly always adopt the pyranose form when conjugated to flavonoids. The stereochemistries of the sugar (D/L and α/β designations) are also potentially variable. However, when conjugated to flavonoids most saccharides adopt a preferred stereochemistry, with glucose, galactose and rhamnose typically occurring as the β-D isomer, and rhamnose and arabinose occurring as the α-L isomer. These preferred stereochemistries are nearly universal, as evidenced by the compounds listed in *The Handbook of Natural Flavonoids*.[1] Of the 1097 flavonol glycosides included in the handbook, there are only six compounds that contradict these generalizations: two examples of an α-D-glucoside, one example of an α-D-galactoside, two examples of a β-L-

arabinoside, and one example of a D-arabinoside (the α/β stereochemistry of the anomeric carbon is unspecified).

Another layer of complication is introduced when disaccharides or trisaccharides are involved. In addition to the above structural issues, the linkage positions between the saccharide monomers must be determined. Linkage isomerism is not uncommon, as in the case of hesperidin and neohesperidin (Figure 1.3), both found in orange juice. These flavanone glycosides are both

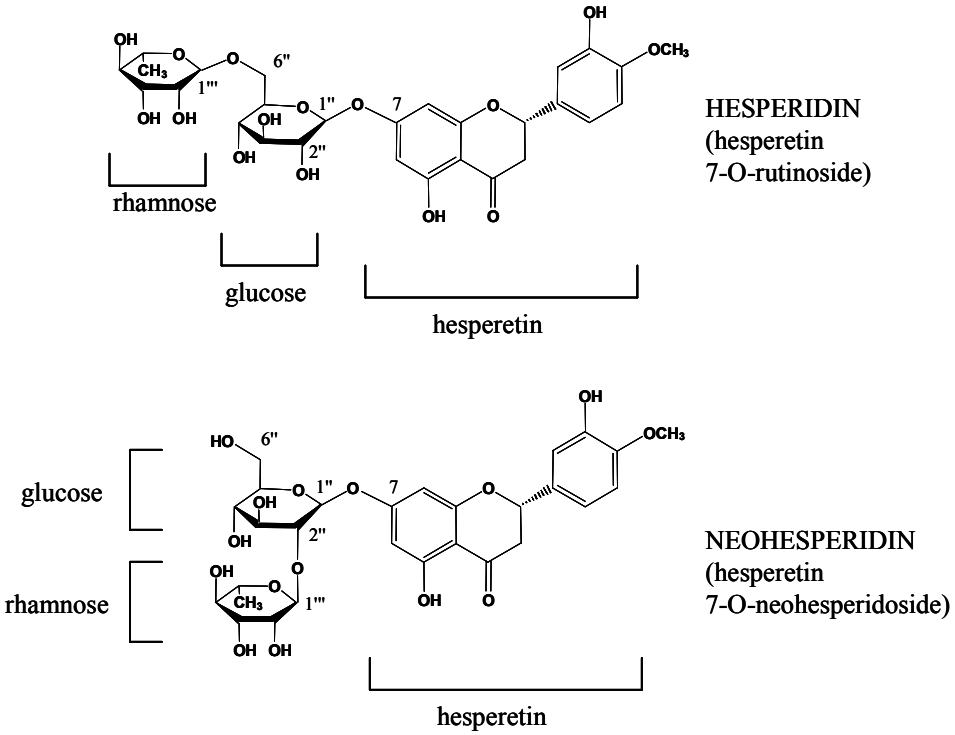


Figure 1.3: Chemical structures of two isomeric flavanone glycosides, hesperidin and neohesperidin. These compounds are linkage isomers, differing only in the linkage between the two saccharide monomers.

based on the aglycon hesperetin, and are glycosylated at the 7 position. In both compounds β-D-glucopyranose is conjugated to the hesperetin aglycon via carbon 1″ of the sugar. α-L-rhamnopyranose is linked to the glucose moiety. The sole difference between the two compounds lies in the intersaccharide linkage. Hesperidin links carbon 1‴ of rhamnose to oxygen 6″ of glucose, while neohesperidin links carbon 1‴ of rhamnose to oxygen 2″ of glucose.

The naming convention for flavonoid glycosides first gives the name of the aglycon on which the structure is based, followed by the glycosylation position, an indication of whether the saccharide is linked through a oxygen or carbon atom, and finally the name of the saccharide (e.g. kaempferol 3-O-glucoside). The saccharide stereochemistry may optionally be included, but since this is strongly dependent on the identity of the saccharide, it will be omitted in this work. The furanose/pyranose distinction will also be omitted except in the case of flavonoid arabinosides. Many flavonoid glycosides also have trivial names, such as "astragalin" for kaempferol 3-O-glucoside. Whenever trivial names for flavonoid glycosides are used, the systematic name will also be given at the first occurrence in each chapter.

1.3 ESTABLISHED METHODS FOR IDENTIFYING FLAVONOID DERIVATIVES

While there are some established methods for flavonoid identification, there are drawbacks to each of them. The gold standard for structural

identification of any organic compound is nuclear magnetic resonance (NMR) spectroscopy. This powerful technique allows the structures of unknown molecules to be deduced with little prior information required. However, NMR has the liability of requiring relatively large amounts (milligram quantities) of very pure samples. This is particularly true for flavonoid compounds, which often require ^{13}C NMR for confident structural assignment.[59,60] Isolation of individual compounds is a difficult and time-consuming process, and some compounds may not be present in sufficient quantities for NMR analysis. In particular, this technique is rarely used to identify flavonoid metabolites because these compounds are produced in such small quantities. Recently significant efforts have been put forth to couple liquid chromatography to NMR spectroscopy in order to combat some of these drawbacks.[60-62] Coupling these two technologies is a significant technical challenge and while some progress has been made, the approach is still uncommon, not least because of the long data acquisition time and high monetary costs (for deuterated solvents) associated with this strategy.[63]

An alternative method based on UV-Vis (ultraviolet-visible) spectroscopy was introduced by Tom Mabry and co-workers.[64] By taking spectra of hundreds of flavonoids and flavonoid glycosides, many strong correlations were found between chemical structure and UV absorption characteristics. Several UV shift reagents, such as sodium acetate and aluminum chloride are systematically used to alter the UV spectra in structurally-dependent ways. Information on the

identity of the flavonoid aglycon and the positions of saccharide groups are possible with this methodology, although the identities of the saccharide groups cannot be deduced in this way. Coupling UV-Vis detection to HPLC analysis is simple and prevents the need for isolating individual compounds. But the complex set of rules and experiments and shift reagents needed to deduce structures makes it somewhat inconvenient, so it would be advantageous to develop a simpler means to obtain the same information.

Mass spectrometry (MS) is a good alternative to both NMR and UV-Vis techniques. MS is more sensitive than traditional NMR by several orders of magnitude, and recent progress in commercialization has made mass spectrometry a routine technique in many laboratories. It is more easily coupled to HPLC than NMR, does not require the use of expensive deuterated solvents, and entails shorter acquisition times. However, full structural analysis of flavonoids has not yet been achieved via MS. Progress has been made toward this end in recent years, but the information obtained is still not as complete as can be achieved by NMR spectroscopy.

Mass spectrometry is a quickly-evolving analytical technique. While a considerable body of mass spectrometric work on flavonoids was done using electron ionization (EI), fast-atom bombardment (FAB) and liquid secondary ion mass spectrometry (LSIMS) techniques,[65] these approaches are now somewhat dated. For example, EI only works on volatile analytes, so much effort went into

finding suitable derivatization methods to volatilize flavonoids to allow them to be analyzed by this technique. More modern approaches to mass spectrometry do not require volatile analytes and have thus replaced EI mass spectrometry except in gas chromatography applications. Currently, the ionization method of choice for flavonoids is electrospray ionization (ESI). Since its adaptation to mass spectrometry in the late 1980's,[66] ESI has become one of the most popular ionization methods and is now a mainstay of most commercial mass spectrometers. Older ionization techniques such as FAB and LSIMS have been largely supplanted by newer methods, and are only encountered on older instruments or in highly specialized applications. The discussion of the current state of MS analysis of flavonoids will thus focus mainly on ESI methodology.

ESI analysis may be performed on either positively-charged or negatively-charged flavonoids. There are advantages and disadvantages to both approaches. Users of negative ion mode ESI report higher sensitivity and avoid the unwanted sodium adducts that often plague positive mode analysis of flavonoids.[67-70] Proponents of positive ion mode report more varied ion fragmentation, which is useful for structural characterization of flavonoids. The incidental sodium adducts sometimes are also studied to provide additional structural information.[57] Under the conditions used in the following studies, it was often impossible to observe non-adducted flavonoids and flavonoid derivatives in positive ESI mode

due to the overwhelming presence of sodium adducts, so negative ion mode was used exclusively for the analysis of unmodified compounds.

Identifying flavonoid aglycons using negative ion mode ESI is usually a fairly simple process when collision-induced dissociation (CID) is employed. Although isomerism prevents identification based on mass alone, a single stage of CID fragmentation is often enough to reveal the structure. The fragmentation of flavonoid aglycons usually yields a highly characteristic set of product ions that can be used to identify the compound.[71-74] For example, Figure 1.4 shows negative ion mode CID spectra of the isomers quercetin and morin (exact mass =

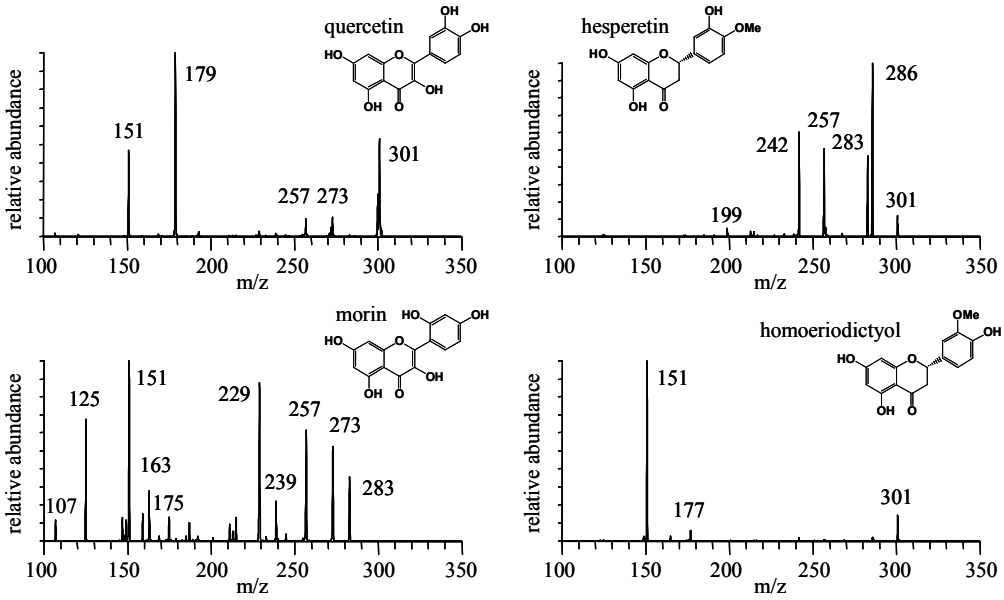


Figure 1.4: Negative ion mode CID MS/MS data for four isobaric flavonoid aglycons of mass 302 Da.

302.04 Da) as well as isobaric compounds hesperetin and homoeriodictyol (exact mass = 302.08 Da). The array of ions yielded by each compound is sufficient to differentiate each one despite the similarity of their structures and their indistinguishable masses.

Identifying the aglycon on which a flavonoid glycoside is based works very much the same way in the case of the ubiquitous O-glycosides. CID of negatively-charged flavonoid O-glycosides results in the neutral loss of the saccharide moieties. If more than one saccharide is present, it may take multiple CID steps to remove all them. Once the bare deprotonated aglycon is left, it will almost always fragment nearly identically to a native aglycon. Thus it is usually possible to identify the aglycon portion of unknown flavonoid glycosides by comparison with a library of aglycon fragmentation patterns. Exceptions to this generalization will be discussed in Chapter 8.

In contrast, limited information is obtained about the saccharide portions of flavonoid O-glycosides. Since the saccharide portions of O-glycosyl flavonoids are typically removed intact via neutral-loss mechanisms, it is possible to determine how many saccharide moieties are present and to learn the mass of each one. But this does not reveal the glycosylation position(s), nor does it provide the exact identities of the individual saccharides. For example, the loss of 162 Da indicates a hexose sugar but does not specify whether that sugar is glucose or galactose. The loss of 132 Da may indicate either arabinose of xylose.

Rhamnose, on the other hand, is the only deoxyhexose known to form natural flavonoid conjugates, so a loss of 146 Da is sufficient to identify this sugar.[75]

C-glycosyl flavonoids follow dissociation pathways involving cross-ring cleavages of the saccharide moiety, resulting in characteristic losses of 90 Da or 120 Da (from glucose).[58] Cross-ring cleavages will be described in more detail in Section 2.4. Dehydration pathways involving the loss of one or more water molecules has also been reported in mass spectral analysis of deprotonated C-glycosyl flavonoids. The systematic differentiation of the 6-C and 8-C glycosylation sites based on MS data has been reported.[58,70,76-78]

Several good review articles on using mass spectrometry to identify flavonoids and flavonoid glycosides have been written. A 2004 review by Cuyckens and Claeys[57] provides more information on all of the aspects described in this section. Summaries of techniques using older MS methods can be found in other review articles.[65,79-81]

1.4 GOALS AND OVERVIEW

This dissertation presents a body of work aimed at developing facile new MS methods for structural characterization of monoglycosyl flavonoids. While much work has already been done using mass spectrometry for this type of application, there are still structural determinations that cannot be made using mass spectrometry alone. This work is geared towards allowing mass

spectrometry to be the sole characterization technique required for full structural analysis of flavonoids and flavonoid glycosides, using methods that are more sensitive and less laborious than either NMR or UV-Vis strategies.

Chapter 2 gives a general description of the methods employed in the subsequent chapters as well as additional technical background. Metal ion complexation is introduced as a novel method to obtain structural information about flavonoids using MS techniques. The use of HPLC data to glean additional evidence of structure is discussed. Finally, the conventions for naming MS fragmentation pathways of flavonoids are provided.

Chapters 3 and 4 describe ways of overcoming two of the primary shortcomings of mass spectrometric identification of flavonoid glycosides. Chapter 3 is concerned with the determination of the glycosylation site of monoglucosyl flavonoids, while Chapter 4 deals with the specific identification of the saccharide moiety. Metal complexation is the key step in providing both pieces of information. These chapters will cover different types of complexes and the ability of each type to provide the desired information.

Chapter 5 discusses the practical implementation of the techniques described in the previous two chapters. The flavonoid glycosides in apple peel, red onions and *Silphium albiflorum* are identified based on these methods. The first two applications were checked for accuracy against previously published studies, providing enough confidence in the method to perform an analysis of

Silphium albiflorum, a plant whose flavonoids have not been previously identified and could not be verified by NMR spectroscopy.

Chapter 6 changes the focus from flavonoid glycosides to flavonoid metabolites. Metal complexes that can identify glucuronidated flavonoid metabolites are discussed. Implementation of these methods is also covered. Human flavonoid metabolites found in urine following the consumption of grapefruit juice and orange juice were identified using these methods. Several of the metabolites have not been previously reported in humans. Metabolites in a cell culture treated with flavonoids were also identified.

Chapter 7 uses the methodology introduced in Chapter 6 to explore the regioselectivity of UDP-glucuronosyltransferase 1A1, an enzyme that is responsible for glucuronidation of flavonoids. An enzymatic synthesis was performed using several flavonoids as substrates for the reaction. The products were identified using only LC-MS methods, particularly the metal complexes found to elucidate the glucuronidation positions of flavonoids. The product distributions of the various substrates allow general trends to be deduced. The success of the metal complexation method also provides confirmation of its efficacy.

Chapter 8 explores a particular problem in the fragmentation behavior of flavonoid glycosides by tandem mass spectrometry. As mentioned earlier, sequential dissociation of flavonoid glycosides usually will yield a fragmentation

spectrum similar to that of the native aglycon after all of the saccharides have been cleaved. This provides a simple method for identifying the aglycon portion of flavonoid glycosides. Chapter 8 explores a few cases in which this does not occur. Implications for the identification of flavonoid glycosides by mass spectrometry are discussed.

Finally, Chapter 9 gives a summary and critical analysis of the research presented in this dissertation. The general capabilities and limitations of the described methods are assessed, and implementation concerns are addressed. The impact of this work on flavonoids research is also evaluated.

1.5 REFERENCES

(1) Harborne JB, Baxter H. *The Handbook of Natural Flavonoids, Vol. 1 & 2*; John Wiley & Sons: Chichester, **1999**.
(2) Siegler DS. *Plant Secondary Metabolism, Part I*; Kluwer Academic: Boston, **1998**.
(3) Kuhnau J. *World Review of Nutrition and Dietetics* **1976**, *24*, 117.
(4) Hertog MGL, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M, Simic BS, Toshima H, Feskens EJM, Hollman PCH, Katan MB. *Archives of Internal Medicine* **1995**, *155*, 381-386.
(5) Sampson L, Rimm E, Hollman PCH, de Vries JHM, Katan MB. *Journal of the American Dietetic Association* **2002**, *102*, 1414-1420.
(6) Chun OK, Chung SJ, Song WO. *Journal of Nutrition* **2007**, *137*, 1244-1252.
(7) Ross JA, Kasum CM. *Annual Review of Nutrition* **2002**, *22*, 19-34.
(8) Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. *American Journal of Medicine* **2002**, *113*, 71-88.
(9) Manthey JA, Guthrie N, Grohmann K. *Current Medicinal Chemistry* **2001**, *8*, 135-153.

(10) Mennen LI, Sapinho D, de Bree A, Arnault N, Bertrais S, Galan P, Hercberg S. *Journal of Nutrition* **2004**, *134*, 923-926.
(11) Oleszek W. *Phytochemistry Reviews* **2002**, *1*, 163-166.
(12) Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. *Lancet* **1993**, *342*, 1007-1011.
(13) Kyle J, Duthie G. *Natural Product Communications* **2006**, *1*, 1049-1060.
(14) Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP, Gescher AJ. *International Journal of Cancer* **2007**, *120*, 451-458.
(15) Graf BA, Milbury PE, Blumberg JB. *Journal of Medicinal Food* **2005**, *8*, 281-290.
(16) Kanadaswami C, Lee LT, Lee PPH, Hwang JJ, Ke FC, Huang YT, Lee MT. *In Vivo* **2005**, *19*, 895-909.
(17) Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. *Critical Reviews in Food Science and Nutrition* **2005**, *45*, 287-306.
(18) Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. *American Journal of Clinical Nutrition* **2001**, *74*, 418-425.
(19) Middleton E, Kandaswami C, Theoharides TC. *Pharmacological Reviews* **2000**, *52*, 673-751.
(20) Arts ICW, Hollman PCH. *American Journal of Clinical Nutrition* **2005**, *81*, 317s-325s.
(21) Hertog MGL, Feskens EJM, Kromhout D. *Lancet* **1997**, *349*, 699-699.
(22) Knekt P, Jarvinen R, Reunanen A, Maatela J. *British Medical Journal* **1996**, *312*, 478-481.
(23) Yochum L, Kushi LH, Meyer K, Folsom AR. *American Journal of Epidemiology* **1999**, *149*, 943-949.
(24) Arts ICW, Hollman PCH, Feskens EJM, de Mesquita HBB, Kromhout D. *American Journal of Clinical Nutrition* **2001**, *74*, 227-232.
(25) Hirvonen T, Pietinen P, Virtanen M, Ovaskainen ML, Hakkinen S, Albanes D, Virtamo J. *Epidemiology* **2001**, *12*, 62-67.
(26) Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM. *American Journal of Clinical Nutrition* **2002**, *75*, 880-886.
(27) Sesso HD, Gaziano JM, Liu S, Buring JE. *American Journal of Clinical Nutrition* **2003**, *77*, 1400-1408.
(28) Arts ICW, Jacobs DR, Harnack LJ, Gross M, Folsom AR. *Epidemiology* **2001**, *12*, 668-675.
(29) Rimm EB, Katan MB, Ascherio A, Stampfer MJ, Willett WC. *Annals of Internal Medicine* **1996**, *125*, 384-389.
(30) Hertog MGL, Sweetman PM, Fehily AM, Elwood PC, Kromhout D. *American Journal of Clinical Nutrition* **1997**, *65*, 1489-1494.

(31) Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T, Aromaa A. *American Journal of Clinical Nutrition* **2002**, *76*, 560-568.
(32) Keli SO, Hertog MGL, Feskens EJM, Kromhout D. *Archives of Internal Medicine* **1996**, *156*, 637-642.
(33) Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. *Stroke* **2000**, *31*, 2301-2306.
(34) Knekt P, Jarvinen R, Seppanen R, Heliovaara M, Teppo L, Pukkala E, Aromaa A. *American Journal of Epidemiology* **1997**, *146*, 223-230.
(35) Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. *Cancer Causes & Control* **2001**, *12*, 789-796.
(36) Ha JH, Lee JT, Cho IH, Chun KA, Park GE, Choi HC, Lee KY, Kim SH, Suk K, Kim IK, Lee MG. *Phytomedicine* **2007**, *14*, 232-235.
(37) Ju W, Wang X, H. S, Chen W, Belinsky SA, Lin Y. *Molecular Pharmacology* **2007**, *71*, 1381-1388.
(38) Morley KL, Ferguson PJ, Koropatnick J. *Cancer Letters* **2007**, *251*, 168-178.
(39) Ninfali P, Bacchiocca M, Antonelli A, Biagiotti E, Di Gioacchino AM, Piccoli G, Stocchi V, Brandi G. *Phytomedicine* **2007**, *14*, 216-221.
(40) Fang J, Zhou Q, Liu LZ, Xia C, Hu X, Shi X, Jiang BH. *Carcinogenesis* **2007**, *28*, 858-864.
(41) Ma G, Yang C, Qu Y, Wei H, Zhang T, Zhang N. *Chemico-Biological Interactions* **2007**, *167*, 153-160.
(42) Kumar MAS, Nair M, Hema PS, Mohan J, Santhoshkumar TR. *Molecular Carcinogenesis* **2007**, *46*, 231-241.
(43) Yeh TC, Chiang PC, Li TK, Hsu JL, Lin CJ, Wang SW, Peng CY, Guh JH. *Biochemical Pharmacology* **2007**, *73*, 782-792.
(44) Himeji M, Ohtsuki T, Fukazawa H, Tanaka M, Yazaki S, Ui S, Nishio K, Yamamoto H, Tasaka K, Mimura A. *Cancer Letters* **2007**, *245*, 269-274.
(45) Chen KH, Weng MS, Lin JK. *Biochemical Pharmacology* **2007**, *73*, 215-227.
(46) Lentini A, Forni C, Provenzano B, Beninati S. *Amino Acids* **2007**, *32*, 95-100.
(47) Durgo K, Vukovic L, Rusak G, Osmak M, Colic JF. *Food Technology and Biotechnology* **2007**, *45*, 69-79.
(48) Williamson G, Barron D, Shimoi K, Terao J. *Free Radical Research* **2005**, *39*, 457-469.
(49) Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. *Journal of Nutrition* **1998**, *128*, 593-597.
(50) Manach C, Donovan JL. *Free Radical Research* **2004**, *38*, 771-785.

(51) Williamson G, Day AJ, Plumb GW, Couteau D. *Biochemical Society Transactions* **2000**, *28*, 16-22.
(52) Day AJ, Williamson G. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Gross GG, Hemingway RW, Yoshida T, Ed.; Kluwer Academic / Plenum Publishers: New York, **1999**.
(53) Day AJ, Bao YP, Morgan MRA, Williamson G. *Free Radical Biology and Medicine* **2000**, *29*, 1234-1243.
(54) Williamson G. *Phytochemistry Reviews* **2002**, *1*, 215-222.
(55) Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G. *American Journal of Clinical Nutrition* **2004**, *80*, 15-21.
(56) Markham KR. *Techniques of Flavonoid Identification*; Academic Press: London; New York, **1982**.
(57) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
(58) Waridel P, Wolfender JL, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Journal of Chromatography A* **2001**, *926*, 29-41.
(59) Agrawal PK, Markham KR. In *Carbon-13 Nmr of Flavonoids*; Agrawal PK, Ed.; Elsevier: Amsterdam, **1989**.
(60) de Rijke E, Out P, Niessen WMA, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2006**, *1112*, 31-63.
(61) Wolfender JL, Queiroz EF, Hostettmann K. *Magnetic Resonance in Chemistry* **2005**, *43*, 697-709.
(62) Wolfender JL, Ndjoko K, Hostettmann K. *Journal of Chromatography A* **2003**, *1000*, 437-455.
(63) Elipe MVS. *Analytica Chimica Acta* **2003**, *497*, 1-25.
(64) Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, **1970**.
(65) Stobiecki M. *Phytochemistry* **2000**, *54*, 237-256.
(66) Yamashita M, Fenn JB. *Journal of Physical Chemistry* **1984**, *88*, 4451-4459.
(67) Cuyckens F, Claeys M. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 2341-2348.
(68) Rauha JP, Vuorela H, Kostiainen R. *Journal of Mass Spectrometry* **2001**, *36*, 1269-1280.
(69) de Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2003**, *984*, 45-58.
(70) Becchi M, Fraisse D. *Biomedical and Environmental Mass Spectrometry* **1989**, *18*, 122-130.
(71) Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(72) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.

(73) Hughes RJ, Croley TR, Metcalfe CD, March RE. *International Journal of Mass Spectrometry* **2001**, *210/211*, 371-385.
(74) Kuhn F, Oehme M, Romero F, Abou-Mansour E, Tabacchi R. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.
(75) Cuyckens F, Shahat AA, Pieters L, Claeys M. *Journal of Mass Spectrometry* **2002**, *37*, 1272-1279.
(76) Cuyckens F, Ma YL, Pocsfalvi G, Claeys M. *Analusis* **2000**, *28*, 888-895.
(77) Li QM, Van den Heuvel H, Dillen L, Claeys M. *Biological Mass Spectrometry* **1992**, *21*, 213-221.
(78) Bakhtiar A, Gleye J, Moulis C, Fouraste I. *Phytochemical Analysis* **1994**, *5*, 86-89.
(79) Cuyckens F, Ma YL, Pocsfalvi G, Claeys M. *Analusis* **2000**, *28*, 888-895a.
(80) Wolfender JL, Maillard M, Marston A, Hostettmann K. *Phytochemical Analysis* **1992**, *3*, 193-214.
(81) Mabry TJ, Ulubelen A. In *Biochemical Applications of Mass Spectrometry, 1st Supplemental Volume*; Waller GR, Dermer OC, Ed.; Wiley: New York, **1980**.

Chapter 2: Experimental Methods and Theory

2.1 Instrumentation and Techniques

All mass spectrometric experiments described in the following chapters were performed on a quadrupole ion trap (QIT) mass spectrometer, equipped with an electrospray ionization (ESI) source. There are several advantages to using ESI-QIT mass spectrometry in the study of flavonoids and other molecules. ESI is a very gentle ionization technique, allowing analytes to be charged and transferred to the gas phase with very little fragmentation. Thus it is usual to observe the intact analyte ion when using this ionization technique.[1,2] ESI is also gentle enough to transfer non-covalent complexes into the gas phase.[3-7] This is important because many of the methods described in the following chapters rely on the analysis of non-covalent complexes between flavonoids and metal ions. A harsher ionization method would break apart such complexes, rendering them useless for subsequent analysis.

A particular strength of the QIT mass analyzer that will be heavily exploited in the following chapters is the facile implementation of multiple-stage tandem mass spectrometry, or MS^n. It has been established experimentally that operating a QIT with a low level of bath gas (~10^{-3} Torr) in the trap increases the efficiency of ion trapping and transmission.[8-10] The presence of this gas also provides the basis for collision-induced dissociation (CID), a process involving

the excitation of selected ions in the trap, low-energy collisions with the bath gas molecules, and subsequent fragmentation of the ions.[9-11] In a QIT, this process can be repeated several times, so as to trace a specific precursor ion through many successive fragmentation stages. A great deal of information about the structure of unknown analytes may be obtained in this manner. CID is achieved by applying a supplementary radio frequency (RF) waveform to the endcaps of the ion trap to cause increased ion motion and therefore more energetic collisions between the ions and the bath gas.[9,10] The energy imparted to ions is not controlled directly, but is instead governed by the applied RF voltage. This voltage is often reported as a percentage of a maximum 5 V_{p-p}, normalized for the mass of the ion being dissociated (as more massive ions require more energy to fragment to the same extent as less massive ions).[12] In most places in the following chapters, the collision energy used in experiments will be reported simply as this percentage.

High-performance liquid chromatography (HPLC) is another method that is employed heavily in this research. HPLC is a popular tool for automated separation of mixtures. Reversed-phased chromatography, the type of chromatography used in these experiments, employs a hydrophobic column that is eluted using a mobile phase gradient that begins with a highly aqueous solvent mixture that becomes more organic over time. Analyte molecules are differentially retained on the column according to their hydrophobicity, such that

non-polar components are retained on the column longer than polar components.[13] A well-chosen method comprising proper selection of column, mobile phase solvents, and gradient will allow even very similar molecules to be separated. In these experiments, the HPLC apparatus is coupled directly to the mass spectrometer so that the analytes can be mass-analyzed in real time, as they elute from the column.

Solid phase extraction (SPE) is a sample preparation technique that is closely related to HPLC, and is used in many of the experiments described in the following chapters. It allows the partial purification of analytes from complex mixtures, including biological matrices. In reversed-phased SPE, a disposable column packed with a hydrophobic stationary phase is used. The sample mixture is loaded onto the column in a high-aqueous solvent so that the analyte of interest will be retained on the stationary phase. The column is first washed with a weak (mostly aqueous) solvent to remove weakly-retained matrix molecules, then a stronger (mostly organic) solvent is used to elute the analyte of interest, leaving irreversibly-bound components on the disposable column.[14] SPE is useful because it reduces the presence of very hydrophilic (e.g. salts) and hydrophobic compounds from the mixture, resulting in a simpler sample mixture for analysis. It can also be used to condense low-concentration analytes into a smaller sample volume.

2.2 METAL COMPLEXATION OF FLAVONOIDS FOR STRUCTURAL CHARACTERIZATION

One of the primary goals of this research is to fill in some of the aforementioned gaps (discussed in Section 1.3) in the capability of mass spectrometry to provide complete structural characterization of flavonoid glycosides. The most important obstacles are the determination of the glycosylation site(s) and the identity of the saccharide(s). Metal complexation was seen as a promising approach to overcome these obstacles. Many research groups have used metal complexation as a means of altering the fragmentation pathways of compounds analyzed by mass spectrometry.[15-24] These complexes often provide more varied fragmentation than underivatized analytes. Metal complexation techniques are particularly suited to flavonoids, which have natural chelation properties and are known to spontaneously form complexes with various metal ions including iron, copper and aluminum.[25-33]

In recent years, much effort has been devoted to the development of metal complexation mass spectrometry methods for the structural elucidation of flavonoid glycosides.[34-45] One of the early observations was that the degree of glycosylation plays a major role in the type of metal complexes required to solve a particular structural problem. In particular, methods that proved effective for diglycosyl flavonoids did not work for monoglycosyl flavonoids, and vice versa. Thus, efforts were divided by flavonoid structure. There is precedent for this type

of division in the work of Leary and co-workers, who have used metal complexation to differentiate isomeric saccharides. They have reported that different complexes are required for differentiation of hexoses, hexosamines and N-acetylated hexosamines.[15-17] The focus of this work is accordingly limited to the monoglycosyl flavonoids. Similar similar methods for diglycosyl flavonoids have been reported.[35-39]

The formation of the complexes simply entails mixing a purified flavonoid glycoside and a metal salt in a 1:1 ratio in methanol (a good solvent for ESI methods). The complexes rapidly self-assemble, and the reaction mixture can be infused directly into the mass spectrometer without clean-up or purification steps. The standard concentration used in the following studies is 10 μM flavonoid glycoside and 10 μM metal salt. Although ESI is a soft ionization source, the spray parameters can be altered to make the process more or less gentle. Very gentle conditions are preferred for this work due to the fragile nature of the non-covalent complexes. It was experimentally determined that the ESI gas flow rates (sheath gas and auxiliary gas) are most important for obtaining optimal signal from metal/flavonoid glycoside complexes. These gas flows were kept as low as possible, taking care not to deteriorate the performance of the ESI source. Because the needle position is routinely altered as part of signal optimization, the sheath and auxiliary gas flows must also be optimized on a day-to-day or run-to-run basis.

Purified flavonoid glycosides are not available for most practical applications, so it was necessary to devise a means of coupling metal complexation with LC-MS separations of mixtures. Attempts were made to inject the reaction mixture containing flavonoid glycosides and metal salts onto the HPLC column, but the complexes do not survive chromatography. Instead, an effective post-column complexation method was devised, illustrated in Figure 2.1. In this approach, the analyte is injected as usual onto the HPLC column. Between the UV detector and the mass spectrometer (or between the column and the mass spectrometer, in applications not employing a UV detector), a methanolic solution of the metal salt (and auxiliary ligand, when necessary) is added to the column

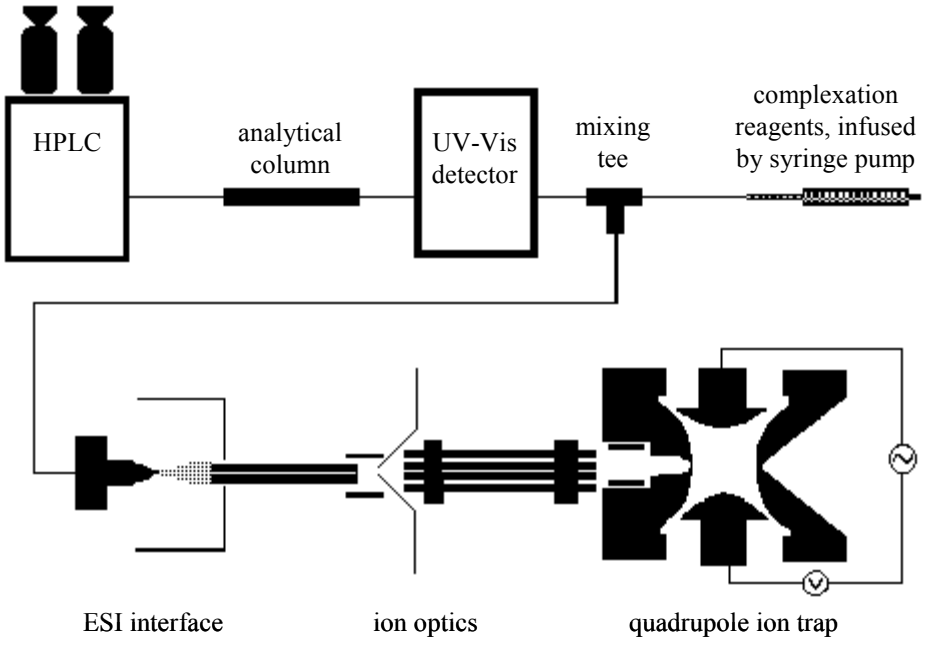


Figure 2.1. Schematic of the instrumental set-up for LC-MS with post-column metal complexation.

effluent via a tee connection. The flow of the post-column reagent(s) is controlled by a syringe pump. The flavonoid analytes and post-column reagents mix at the tee, allowing the complexes to form as analytes elute from the column. These complexes can be analyzed in real-time in the same manner as the directly-infused complexes.

The exact structures of the metal complexes are unknown as is the precise mechanism by which they promote different fragmentation pathways for very similar flavonoid glycosides. However, work by Clowers and Hill has provided insight on both of these problems.[46] Their research used a hybrid ion mobility-quadrupole ion trap mass spectrometer to study the metal complexes of flavonoid diglycosides discovered by Zhang and Brodbelt[38] which have been shown to provide similar structural determinations as the complexes described in this dissertation. Ion mobility spectrometry separates ions based on size/charge ratio rather than the mass/charge ratio involved with mass spectrometry. Metal complexes of isomeric flavonoid diglycosides were determined to have distinguishable mobilities, indicating that sizes and shapes or these complexes were different despite having the same mass. Moreover, some complexes such as a sodium complex of the flavanone diglycoside hesperidin exhibited bimodal mobility distributions, indicating two different conformations.[46] It is plausible that the metal complexes of isomeric flavonoid glycosides adopt conformations that promote distinct fragmentation patterns, allowing isomer differentiation that

is impossible with unmodified flavonoid glycosides. Some complexes may have more than one distinct geometry, so the different fragmentation patterns may also be the result of different proportions of conformations among isomeric metal complexes. Some molecular modeling of the metal complexes of flavonoid diglycosides has also been performed, providing further evidence that isomeric complexes have different stable conformations.[36,37]

2.3 HPLC Retention Time Analysis for Characterization of Flavonoids

In LC-MS applications, the retention time of various analytes may be used in addition to the MS data for compound identification. In the simplest form, this entails comparing the retention time of an authenticated standard to those of the various analytes in an unknown sample. If a sample component matches the standard in terms of retention time and mass, then it is strongly indicated that the two are the same compound. However, retention time analysis can also be used in the absence of standards, as flavonoid structure is known to track systematically with retention time. In reversed-phase HPLC, it has been reported that flavonoid diglycosides elute before monoglycosides, which elute before aglycons. For isomer pairs with the same aglycon and glycosylation position, flavonoid rutinosides elute before flavonoid neohesperidosides, and galactosides before glucosides.[47] Trends of this nature will be used confirm, and in some cases,

identify flavonoid components from botanical and biological samples. A more complete treatment of this approach is given in Section 7.3.2.

2.4 Nomenclature for Fragment Ions of Flavonoid Aglycons, Flavonoid Glycosides and Metal Complexes

As these studies deal extensively with the fragmentation of flavonoids and flavonoid glycosides, a brief overview of the naming conventions used for flavonoid fragment ions is warranted. Flavonol, flavone and flavanone aglycons undergo two major types of CID fragmentation: small molecule losses and retro Diels-Alder cleavages.[48] Small molecule losses involve the removal of a few atoms from the flavonoid skeleton, often H_2O, CO or CO_2.[48,49] The retro Diels-Alder fragments involve the cleavage of two bonds of the C ring, resulting in complementary fragments containing an intact A ring or B ring.[50-52] Claeys and co-workers have proposed a notation for such fragment ions, in the form ${}^{i,j}A^{+/-}$ and ${}^{i,j}B^{+/-}$, where *i* and *j* denote the numbers of the two broken bonds of the C ring, A or B denotes that the fragment ion contains an intact A or B ring, and either a + or – sign indicates the charge of the ion.[50] For saccharide cleavages of flavonoid glycosides, Domon and Costello notation for glycoconjugates is used.[53] In this dissertation, the important saccharide cleavage product ions are Y_0, indicating the loss of an intact saccharide moiety cleaved at the glycosidic bond, and ${}^{i,j}X$, which indicates the cross-ring cleavage of a sugar ring, with *i* and *j* indicating the

specific bonds of the saccharide ring that were cleaved. The Claeys and the Domon and Costello systems are illustrated in Figure 2.2.

New notation has been devised for metal complexes and their fragmentation products. A generic complex may be abbreviated as [M(II) (FG-H) (Aux)]$^{+}$. In this example, FG stands for any flavonoid glycoside. M refers to a generic metal ion, in this instance in the +2 oxidation state. The chemical symbol for the metal will be used in place of M when specificity is desired. Some of the

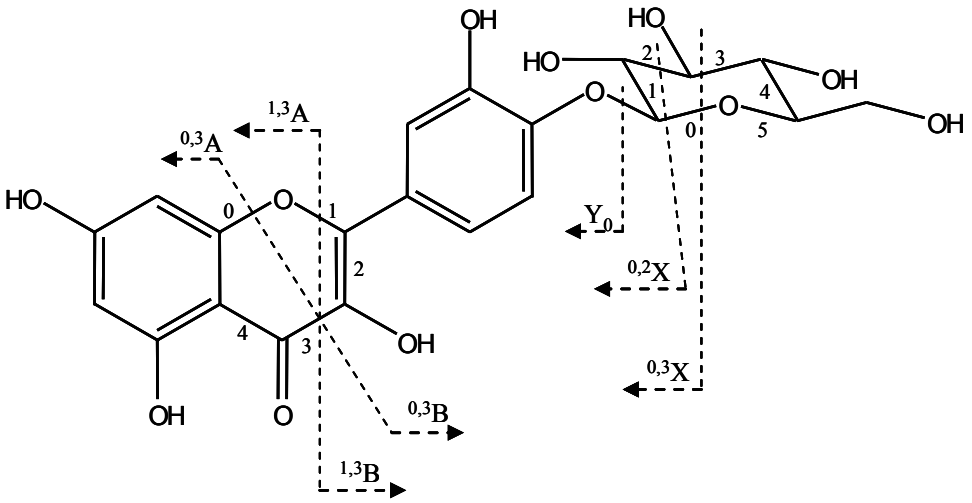


Figure 2.2. Shorthand nomenclature for some common flavonoid fragmentation pathways, using quercetin 4′-O-glucoside as an example. Arrow direction indicates the portion of the molecule that retains the charge after fragmentation. A and B ions illustrate retro Diels-Alder fragmentation, using notation introduced by Claeys and co-workers.[50] X ions illustrate cross-ring saccharide cleavages. X and Y ions follow Domon and Costello naming conventions for glycoconjugate fragment ions.[53] The bond numbering schemes for both systems are indicated.

complexes employ neutral auxiliary ligands, generically abbreviated as Aux, though the actually ligand may be named in specific applications. -H indicates the loss of a hydrogen nucleus, i.e. deprotonation. Certain types of CID fragment ions of such metal complexes occur often enough to warrant abbreviation. –FG indicates the loss of an intact flavonoid glycoside, -Agl indicates the loss of the aglycon portion of a flavonoid glycoside, and -Aux represents the loss of an auxiliary ligand. –Hex and –Pent refer to losses of unspecified hexose or pentose moieties (corresponding to the Y_0 ion of uncomplexed flavonoid glycosides). Standard abbreviations will be used for specific saccharide losses; hence -Glc, -Gal, -Rha, -Ara, -Xyl and -GlcA, represent losses of glucose, galactose, rhamnose, arabinose, xylose and glucuronic acid moieties, respectively. Cross-ring saccharide cleavages (corresponding to the X ions of uncomplexed flavonoid glycosides) will be indicated by the specific mass losses, such as -90 Da, -120 Da, etc. Other abbreviated fragment ions will be explained as they appear unless obvious (e.g. $-H_2O$). Precursor ions in CID spectra are indicated by an asterisk (*).

2.5 REFERENCES

(1) Gabelica V, De Pauw E. *Mass Spectrometry Reviews* **2005**, *24*, 566-587.
(2) Vestal ML. *Chemical Reviews* **2001**, *101*, 361-375.
(3) Pramanik BN, Bartner PL, Mirza UA, Liu YH, Ganguly AK. *Journal of Mass Spectrometry* **1998**, *33*, 911-920.

(4) Tozuka Z, Kaneko H, Shiraga T, Mitani Y, Beppu M, Terashita S, Kawamura A, Kagayama A. *Journal of Mass Spectrometry* **2003**, *38*, 793-808.
(5) Brodbelt JS. *International Journal of Mass Spectrometry* **2000**, *200*, 57-69.
(6) Beck JL, Colgrave ML, Ralph SF, Sheil MM. *Mass Spectrometry Reviews* **2001**, *20*, 61-87.
(7) Henderson W, Evans C. *Inorganica Chimica Acta* **1999**, *294*, 183-192.
(8) Stafford GC, Kelley PE, Syka JEP, Reynolds WE, Todd JFJ. *International Journal of Mass Spectrometry and Ion Processes* **1984**, *60*, 85-98.
(9) Bier ME, Schwartz JC. In *Electrospray Ionization Mass Spectrometry*; Cole RB, Ed.; Wiley: New York, **1997**.
(10) Todd JFJ. In *Practical Aspects of Ion Trap Mass Spectrometry, vol. 1*; March RE, Todd JFJ, Ed.; CRC Press: Boca Raton, **1995**.
(11) Sleno L, Volmer DA. *Journal of Mass Spectrometry* **2004**, *39*, 1091-1112.
(12) Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.
(13) Niessen WMA. *Liquid Chromatography-Mass Spectrometry*; Marcel Dekker: New York, **1999**.
(14) Thurman EM, Mills MS. *Solid Phase Extraction: Principles and Practice*; Wiley: New York, **1998**.
(15) Smith G, Leary JA. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 953-957.
(16) Gaucher SP, Leary JA. *Analytical Chemistry* **1998**, *70*, 3009-3014.
(17) Desaire H, Leary JA. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 1086-1094.
(18) Basso E, Chilin A, Guiotto A, Traldi P. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 2781-2787.
(19) Ho YP, Huang PC, Deng KH. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 114-121.
(20) Asam MR, Glish GL. *Journal of the American Society for Mass Spectrometry* **1997**, *8*, 987-995.
(21) Vaisar T, Gatlin CL, Rao RD, Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2001**, *36*, 306-316.
(22) Carlesso V, Fournier F, Tabet JC. *European Journal of Mass Spectrometry* **2000**, *6*, 421-428.
(23) Cheguillaume G, Buchmann W, Desmazieres B, Tortajada J. *Journal of Mass Spectrometry* **2004**, *39*, 368-377.
(24) Hayen H, Alvarez-Grima MM, Debnath SC, Noordermeer JWM, Karst U. *Analytical Chemistry* **2004**, *76*, 1063-1068.
(25) Fernandez MT, Mira ML, Florencio MH, Jennings KR. *Journal of Inorganic Biochemistry* **2002**, *92*, 105-111.

(26) Kazazic SP, Butkovic V, Srzic D, Klasinc L. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 8391-8396.
(27) Soczynska-Kordala M, Bakowska A, Oszmianski J, Gabrielska J. *Cellular & Molecular Biology Letters* **2001**, *6*, 277-281.
(28) de Souza RFV, Sussuchi EM, De Giovani WF. *Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry* **2003**, *33*, 1125-1144.
(29) de Souza RFV, De Giovani WF. *Spectrochimica Acta Part A* **2005**, *61*, 1985-1990.
(30) Weber G. *Chromatographia* **1988**, *26*, 133-138.
(31) Moridani MY, Pourahmad J, Bui H, Siraki A, O'Brien PJ. *Free Radical Biology and Medicine* **2003**, *34*, 243-253.
(32) Deng HT, Van Berkel GJ. *Journal of Mass Spectrometry* **1998**, *33*, 1080-1087.
(33) Hollman PCH, vanTrijp JMP, Buysman MNCP. *Analytical Chemistry* **1996**, *68*, 3511-3515.
(34) Satterfield M, Brodbelt JS. *Analytical Chemistry* **2000**, *72*, 5898-5906.
(35) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(36) Zhang JM, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 139-151.
(37) Zhang JM, Wang JM, Brodbelt JS. *Journal of Mass Spectrometry* **2005**, *40*, 350-363.
(38) Zhang JM, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1761-1770.
(39) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(40) Pikulski M, Wilson JJ, Aguilar A, Brodbelt JS. *Analytical Chemistry* **2006**, *78*, 8512-8517.
(41) Pikulski M, Aguilar A, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2007**, *18*, 422-431.
(42) Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
(43) Davis BD, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1883-1890.
(44) Davis BD, Needs PW, Kroon PA, Brodbelt JS. *Journal of Mass Spectrometry* **2006**, *41*, 911-920.
(45) Bai Y, Song FR, Chen ML, Xing JP, Liu ZQ, Liu SY. *Analytical Sciences* **2004**, *20*, 1147-1151.
(46) Clowers BH, Hill HH. *Journal of Mass Spectrometry* **2006**, *41*, 339-351.
(47) Robards K, Antolovich M. *Analyst* **1997**, *122*, 11R-34R.
(48) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
(49) March RE, Miao XS. *International Journal of Mass Spectrometry* **2004**, *231*, 157-167.

(50) Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(51) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
(52) Wu W, Yan CY, Li L, Liu ZQ, Liu SY. *Journal of Chromatography A* **2004**, *1047*, 213-220.
(53) Domon B, Costello CE. *Glycoconjugate Journal* **1988**, *5*, 397-409.

Chapter 3: Determination of the Glycosylation Site of Monoglucosyl Flavonoids Using Metal Complexation and Tandem Mass Spectrometry

3.1 INTRODUCTION

Much research has been devoted to the structural elucidation of flavonoids and their derivatives by mass spectrometry.[1-5] But mass spectrometric analysis has not yet reached the point where *de novo* identification of flavonoids is possible. Analysis of flavonoids in plant extracts,[6-12] foodstuffs,[13-18] and human biological fluids[19-21] have been reported, but the identity of flavonoids in these complex matrices generally must be confirmed by comparison to commercial standards. Otherwise only tentative identifications can be made, particularly in terms of saccharide location and identity, unless supplementary analytical methods are employed, such as UV-Vis or NMR spectroscopy.

Some significant progress has been made towards systematic structural characterization of flavonoids by mass spectrometry. As noted in Chapter 1, the fragmentation pathways of flavonoid aglycons obtained by tandem mass spectrometry are well-documented.[22-26] In addition, commonly encountered diglycosyl flavonoids can often be distinguished by their fragmentation pathways.[27-31] For less common flavonoid glycosides, methods have recently been proposed for determining the saccharide identity,[32] as well as the linkage order.[27] Furthermore, distinctive patterns have been reported for methoxylated

flavonoids[33,34] and for chlorinated and nitrated isoflavonoids.[35] Hydrogen/deuterium exchange has also been explored as a method for flavonoid isomer differentiation.[36]

The problem of determining the glycosylation site of monoglycosyl flavonoids has also been addressed. The general fragmentation differences between flavonoid glycosides bonded through carbon versus oxygen atoms have been established,[3] along with differentiation of 6-C-glycosyl and 8-C-glycosyl flavonoids.[3,10,37-39] However, no one had proposed a robust universal mass spectrometric method for determining five of the most common glucosylation positions encountered for the monoglucosyl flavonoids: attachment through oxygen at position 3, 4′, or 7; or through carbon at position 6 or 8. The following methods for achieving this latter goal based on metal complexation and tandem mass spectrometry have been published.[40] Subsequent to this, another mass spectrometry method for the systematic differentiation of flavonoid O-glycosides was reported by Cuyckens and Claeys.[41] Their method involves high-energy collision-induced dissociation (CID) on a hybrid magnetic sector / time-of-flight mass spectrometer, so it is non-competitive with the low-energy CID methods described herein.

A number of groups have studied metal complexes by mass spectrometry and found that the fragmentation pathways of analytes can be significantly altered, allowing new opportunities for structural determination.[42-58] The

Brodbelt group has shown that metal complexation combined with the use of a neutral auxiliary ligand dramatically increases sensitivity for detection of flavonoids while also providing a richer array of fragments to aid in isomer differentiation.[26,30,31,59] In this study, new metal complexation strategies were developed for resolving many isomeric monoglucosyl flavonoids. The resulting fragmentation pathways were remarkably consistent based on the position of the glucose moiety, providing a simple means for determining the glycosylation site of flavonoid glucosides. Fourteen flavonoid glucosides are included in this study (Table 3.1), involving glycosylation at five different positions on the flavonoid skeleton. The flavone, flavonol, and flavanone classes are represented by the sample compounds. Structures of the parent aglycons were shown in Figure 1.2.

Table 3.1. Flavonoid glucosides employed in this study

compound	trivial name	molecular weight
kaempferol 3-O-glucoside	astragalin	448
quercetin 3-O-glucoside	isoquercitrin	464
isorhamnetin 3-O-glucoside	—	478
syringetin 3-O-glucuoside	—	508
luteolin 4′-O-glucuoside	juncein	448
quercetin 4′-O-glucuoside	spiraeoside	464
apigenin 7-O-glucuoside	cosmetin	432
naringenin 7-O-glucuoside	prunin	434
luteolin 7-O-glucuoside	cynaroside	448
quercetin 7-O-glucuoside	quercimeritrin	464
apigenin 6-C-glucuoside	isovitexin	432
luteolin 6-C-glucuoside	homoorientin	448
apigenin 8-C-glucuoside	vitexin	432
luteolin 8-C-glucuoside	orientin	448

3.2 EXPERIMENTAL

All experiments were performed with an LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA) with an electrospray ionization (ESI) source. The ESI flow rate was 5 μL/min, and the ion injection time was typically 10 msec for full scans and 50 msec for MS/MS and MS^3 experiments. 100 microscans were averaged for each spectrum. The mass spectrometer was tuned for maximum intensity of the ion of interest. Protonated flavonoid glucosides, metal complexes, and sodium adducts were analyzed in the positive ion mode; and deprotonated flavonoid glucosides were analyzed in the negative ion mode. The needle voltage was set to +5 kV and a heated capillary temperature of 200 °C was used. The sheath gas and auxiliary gas flow rates were optimized manually on a daily basis, while the ion optics were optimized using the Autotune feature of the LCQ software.

Quercetin 3-O-glucoside, quercetin 3-O-galactoside, syringetin 3-O-glucoside, syringetin 3-O-galactoside, quercetin 4′-O-glucoside, naringenin 7-O-glucoside, kaempferol 7-O-neohesperidoside and luteolin 6-C-glucoside were purchased from Extrasynthèse (Genay, France). Kaempferol 3-O-glucoside, isorhamnetin 3-O-glucoside, luteolin-4′-O-glucoside, apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin 8-C-glucoside, apigenin 6-C-glucoside, luteolin 8-C-glucoside and quercetin 3-O-rhamnoside were purchased from Indofine (Somerville, NJ). Quercetin-7-O-glucoside, quercetin 3-O-arabinofuranoside and

quercetin 3-O-xyloside were purchased from Apin Chemicals (Abingdon, UK). Quercetin 3-O-rutinoside was purchased from Sigma-Aldrich (St. Louis, MO). Certified ACS Spectranalyzed® methanol was purchased from Fisher Scientific (Pittsburgh, PA). $CoBr_2$, $NiBr_2$, $CuBr_2$, $MnCl_2$, $MgCl_2$ and 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen) were purchased from Aldrich (Milwaukee, WI). $CaBr_2$ was purchased from Matheson, Coleman & Bell (Cincinnati, OH). All materials were used without further purification.

Analyte solutions were made in methanol, with the flavonoid glucoside and metal salt each added at 1.0×10^{-5} M except where otherwise noted. The intensities from the collision–induced dissociation (CID) experiments are reported relative to the most intense peak in the spectrum, which is designated as 100%. In collecting MS/MS and MS^3 data, the collision energy was increased until the parent ion was reduced to 5-10% relative abundance. The normalized collision energy range was 18-25%; this corresponds to approximately 0.8-1.1 V applied to the ion trap.[60] The isolation window was typically 2 m/z units, though for some complexes it was necessary to increase the window to as much as 4 m/z units to obtain a stable signal due to the fragile nature of the complexes.[61] All tables list fragment ions down to 2% relative abundance. Post-CID solvent adducts (e.g. $[\text{fragment ion} + CH_3OH]^+$) were apparent in several instances, but as their appearance is dependent on the background pressure and the contents of the trap,

they are not regarded as reliable diagnostic products, and are generally excluded from the analysis.

3.3 Results and Discussion

3.3.1 General Results

Until the recent development and application of metal complexation strategies, flavonoids have generally been analyzed as protonated or deprotonated species by mass spectrometry. Greater sensitivity has been reported in the negative ion mode than in the positive ion mode,[10,62-64] a factor attributed to the substantial acidities of flavonoids and the ease of deprotonation due to their multiple hydroxyl groups. However, the CID spectra of deprotonated flavonoid glucosides generally show few fragments, and are therefore not particularly useful for isomer differentiation. The most successful and confident isomer differentiation results in unique fragment ions for different isomers, although sometimes differences in ratios or distributions of fragment ions may be considered. The goal was to develop a simple but robust method based on metal complexation and CID for differentiation of flavonoid glucosides. Several metals, including Ca(II), Mg(II), Mn(II), Co(II), Ni(II), and Cu(II), were evaluated for their ability to form useful complexes that give diagnostic fragmentation patterns upon CID. These results are discussed in detail in this chapter.

Studies have suggested that certain structural features are required for metal chelation by flavonoids. Proposed chelation sites include the 4-keto and 5-OH groups, the 4-keto and 3-OH groups, and the 3′-OH and 4′-OH groups.[59,65-68] It is possible that more than one of these sites is active for flavonoids containing multiple binding groups. Every flavonoid glucoside in this study contains at least the 4-keto and 5-OH groups, allowing them to form metal complexes. Flavonoids lacking suitable chelation sites are unlikely to be amenable to metal complexation.

The complexes were produced from methanolic solutions containing 1:1 flavonoid glucoside/metal salt. No pH adjustment was performed on the analyte solutions. Under these conditions, the flavonoid glucosides formed 1:1 and 2:1 analyte/metal complexes of the type $[M(II)\ (FG-H)]^+$ and $[M(II)\ (FG-H)\ (FG)]^+$. Additionally, larger complexes were occasionally observed, including 3:1, 3:2, 4:1, and 4:2 analyte/metal stoichiometries. Some of these stoichiometries have been previously reported for copper and iron complexes of flavonoids.[69] Figure 3.1 shows the full scan mass spectrum of isovitexin and Ca(II), which form complexes of several different stoichiometries. The intensities of the 1:1 analyte/metal complexes are often very low, and CID results in complicated mass spectra that often do not assist in compound identification. In contrast, the 2:1 complexes are more intense, and they give simple CID spectra with easily-assigned fragments and a variety of dissociation pathways for structural determination. A possible structure for the 2:1 complexes is shown in Schemes

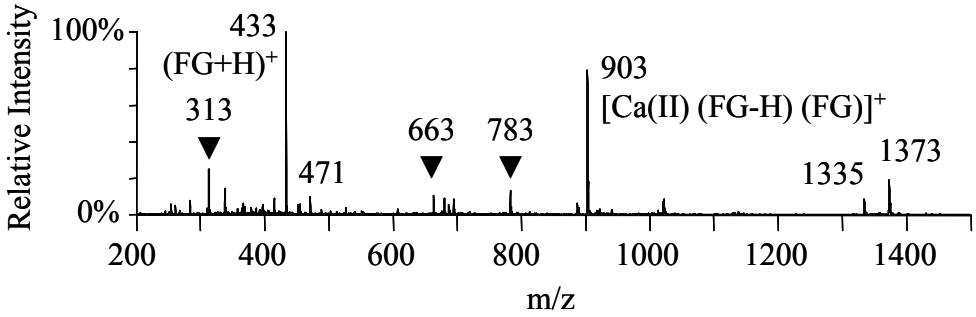
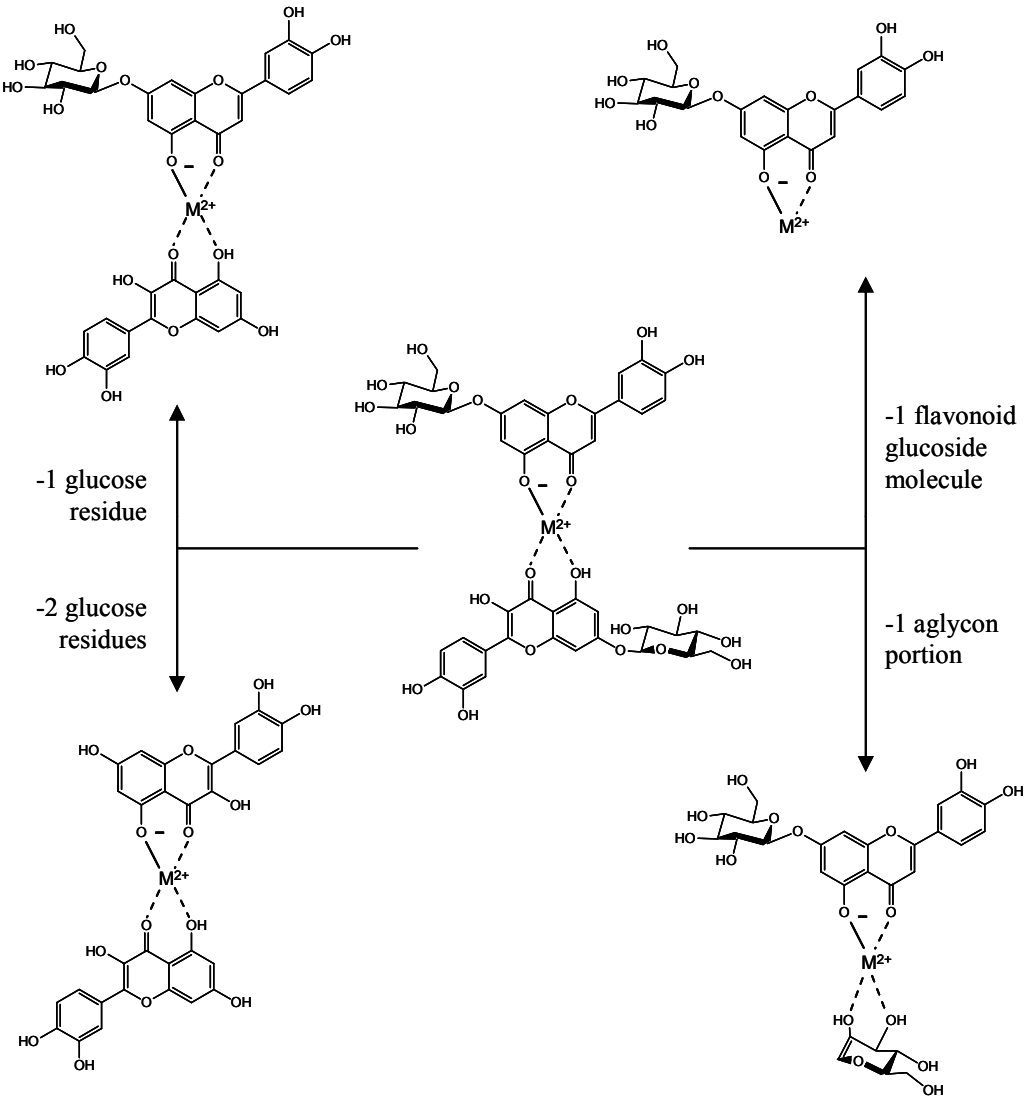


Figure 3.1. Full scan spectrum of isovitexin and Ca(II). FG = isovitexin. 0,2 cross-ring cleavages of m/z 433 and 903 are represented by ▼. Other minor ions include 471: $[Ca(II)\ (FG\text{-}H)]^+$; 1335: $[Ca(II)\ (FG\text{-}H)\ (FG)_2]^+$; and 1373: $[2\ Ca(II)\ (FG\text{-}H)_3]^+$.

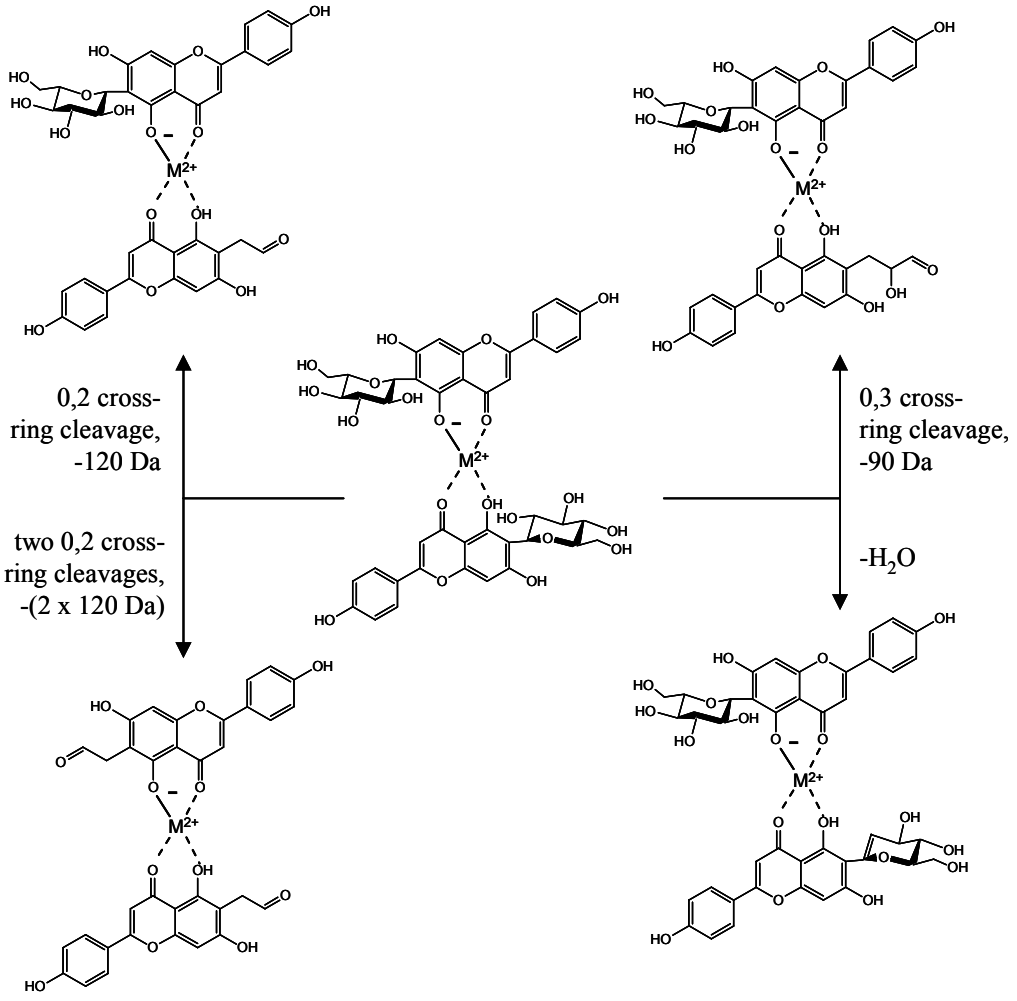
3.1 and 3.2. The complexes involve a doubly-charged metal ion, one deprotonated flavonoid glucoside and one neutral flavonoid glucoside. The chelation site is speculative and likely to be compound-dependent; and it is even possible that more than one structure forms for some analyte/metal complexes. Likewise, the coordination geometry of the complexes is unknown, though particular metals are known to favor certain geometries over others.

One of the common features of all of the metal complexes, regardless of the type of metal ion, is that the C-glucosides and O-glucosides form complexes that fragment very differently from each other. Typical dissociation routes for O-glucosyl flavonoid complexes include loss of one or two glucose moieties, loss of an entire flavonoid glucoside molecule, and loss of the aglycon portion of one flavonoid glucoside (Scheme 3.1). These types of fragments are not obtained from C-glucosyl flavonoid complexes, which instead follow dissociation



Scheme 3.1. Speculative structures of fragment ions commonly observed for metal complexes of O-glucosyl flavonoids (luteolin 7-O-glucoside shown as example).

pathways involving dehydration and cross-ring cleavages of the glucose moiety, resulting in losses of 90 Da or 120 Da (Scheme 3.2). Figure 2.2 more clearly shows which bonds are broken as the result of various cross-ring cleavages.



Scheme 3.2. Speculative structures of fragment ions commonly observed for metal complexes of C-glucosyl flavonoids (isovitexin shown as example).

These types of losses are well-known for C-glycosyl flavonoids, and were discussed in detail by Waridel et al.[38] The loss of one or more water molecules observed with some C-glycosyl flavonoid complexes has also been reported in

mass spectral analysis of deprotonated C-glycosyl flavonoids[10,37,38] and alternate structures for these ions have been proposed.[37]

A direct comparison of the CID mass spectra of deprotonated flavonoid glucosides and flavonoid glucoside/metal complexes illustrates the advantage of the metal complexation strategy. Figure 3.2 shows CID mass spectra obtained for three isomeric deprotonated flavonoid glucosides and the corresponding Mg(II) complexes. The three compounds are all quercetin derivatives, with the glucose moiety attached at either the 3, 4′, or 7 position on the flavonoid skeleton. The

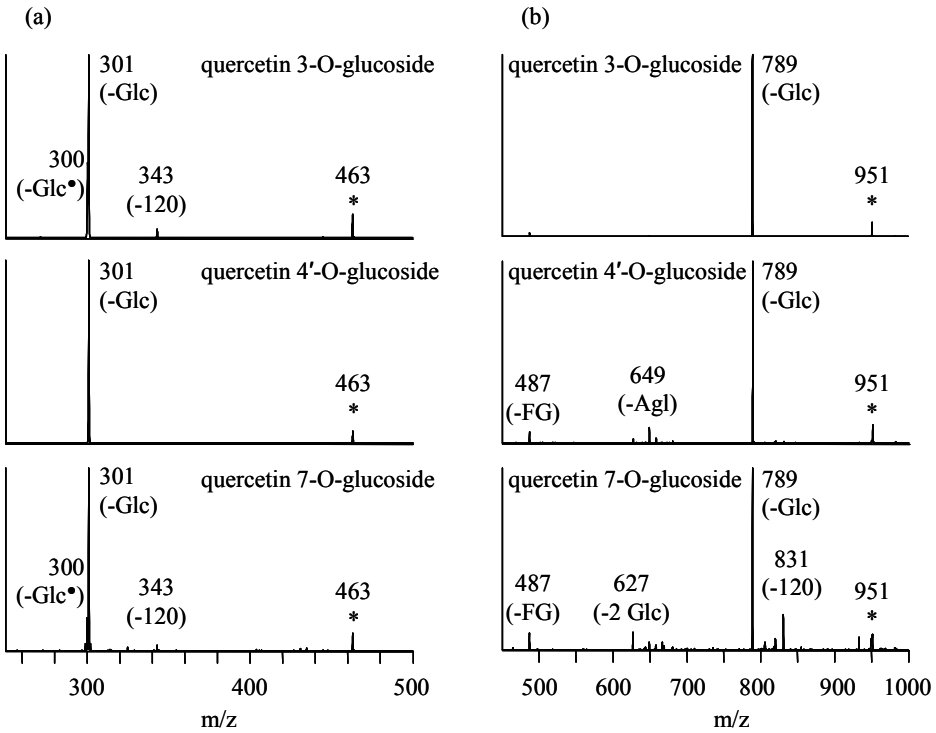


Figure 3.2. CID spectra of quercetin monoglucosides, MW=464. (a) Fragments obtained from deprotonated analytes. (b) Fragments obtained from $[Mg(II)\ (FG\text{-}H)\ (FG)]^+$.

dominant fragmentation pathway of the deprotonated flavonoid glucosides is the loss of the glucose moiety, -162 Da (Figure 3.2 a). Both quercetin-3-O-glucoside and quercetin-7-O-glucoside undergo a radical loss of the glucose moiety (-163 Da) and a cross-ring saccharide cleavage resulting in loss of 120 Da, but the latter pathway results in fragment ions with less than 5% relative intensity. The few fragmentation pathways of deprotonated flavonoid glucosides are insufficient for differentiating these isomers, which differ only by the position of glucosylation. Similarly, protonated flavonoid glucosides and sodium adducts of the form $[Na\ (FG)]^+$ and $[Na\ (FG)_2]^+$ produce few fragments upon dissociation, and in many cases isomer differentiation is not possible (data not shown). In contrast, alkaline earth metal complexes show greater variety in their dissociation pathways. The CID mass spectra of the magnesium complexes exhibit distinctive features for each of the three isomers (Figure 3.2 b). The major fragmentation pathway is loss of the glucose moiety for all three complexes. This is the only significant fragment observed for the quercetin 3-O-glucoside complex. The $[Mg(II)\ (FG - H)\ (FG)]^+$ complex of quercetin 4′-O-glucoside additionally undergoes loss of an aglycon unit and elimination of one flavonoid glucoside molecule, providing sufficient differentiation from quercetin 3-O-glucoside. The quercetin 7-O-glucoside complex displays a unique ion of m/z 831, corresponding to a 0,2 cross-ring cleavage of one glucose moiety (-120 Da, 20% intensity). All three complexes display unique CID mass spectra that allow differentiation of the

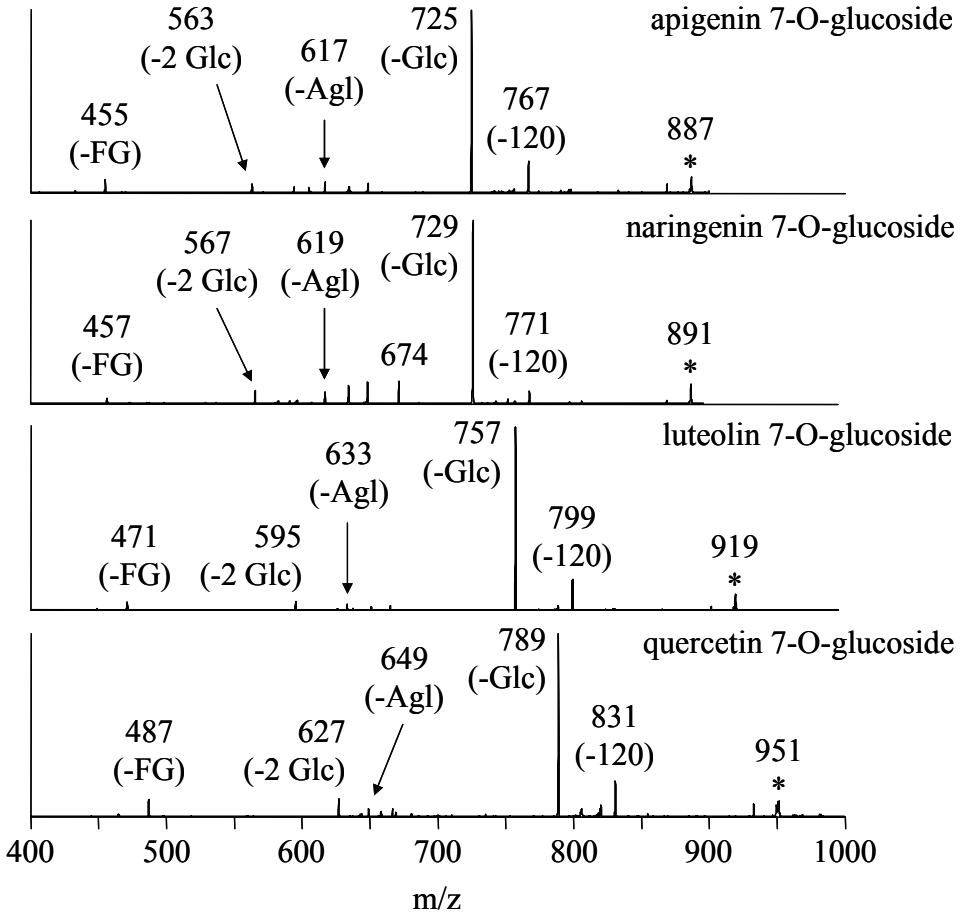


Figure 3.3. CID spectra of [Mg(II) (FG-H) (FG)]$^+$ complexes of 7-O-glucosyl flavonoids. The ion labeled 674 corresponds to ([2 Mg(II) (FG-H)$_2$ (FG)$_2$]$^{2+}$ - FG).

flavonoid glucosides. Furthermore, the pattern of fragments observed for many of the metal complexes is strongly dependent on the site of glucosylation. Figure 3.3 shows the CID mass spectra of the four 7-O-glucosyl flavonoids complexed to Mg(II). The types of fragments and their relative intensities are very similar, despite differences in the aglycon structure. This example demonstrates that the

dissociation patterns of the magnesium complexes are correlated with the glucosylation site, and may be used to determine this information for unknown flavonoid glucosides.

In the following sections, the CID mass spectra of the calcium, magnesium, manganese, cobalt, nickel, and copper complexes are discussed, with an emphasis on the diagnostic utility of the fragmentation patterns for glycosylation site determination.

3.3.2 Calcium Complexes

The list of fragments observed upon CID of the $[Ca(II)\ (FG\text{-}H)\ (FG)]^+$ complexes is shown in Table 3.2. The complexes involving O-glucosyl flavonoids are readily differentiated from those involving C-glucosyl flavonoids. For all of the O-glucosyl flavonoids, the most intense fragment results from the elimination of one glucose residue. In contrast, the complexes of the C-glucosyl flavonoids do not display this loss. Their dominant dissociation pathway is a 0,2 cross-ring cleavage of one glucose moiety, resulting in the loss of 120 Da. Precise identification of the glycosylation site is possible using the strategy outlined in Scheme 3.3. The complexes involving 7-O-glucosyl flavonoids are the only ones to display both the loss of an intact glucose moiety and a 0,2 cross-ring cleavage of one glucose moiety. The 4′-O-glucosyl flavonoids are differentiated from the 3-O-glucosyl flavonoids by minor losses of one flavonoid glucoside

Table 3.2 CID losses from $[Ca(II)\ (FG\text{-}H)\ (FG)]^+$ complexes

a) MS/MS

flavonoid glucoside	-Glc	-FG	-Agl	-2Glc	DC-FG	$(FG+H)^+$	-120	-(2 x 120)	-90
kaempferol 3-O-Glc	100	—	—	—	—	—	—	—	—
quercetin 3-O-Glc	100	—	—	—	—	—	—	—	—
isorhamnetin 3-O-Glc	100	—	—	—	6	—	—	—	—
luteolin 4′-O-Glc	100	8	5	—	25	—	—	—	—
quercetin 4′-O-Glc	100	4	4	—	19	—	—	—	—
apigenin 7-O-Glc	100	3	—	5	—	6	15	—	—
naringenin 7-O-Glc	100	5	—	—	9	—	8	—	—
luteolin 7-O-Glc	100	2	—	6	—	5	15	—	—
quercetin 7-O-Glc	100	4	—	—	—	3	12	—	—
apigenin 6-C-Glc	—	—	—	—	—	—	100	3	8
luteolin 6-C-Glc	—	—	—	—	—	—	100	2	8
apigenin 8-C-Glc	—	—	—	—	—	—	100	5	6
luteolin 8-C-Glc	—	—	—	—	—	—	100	—	6

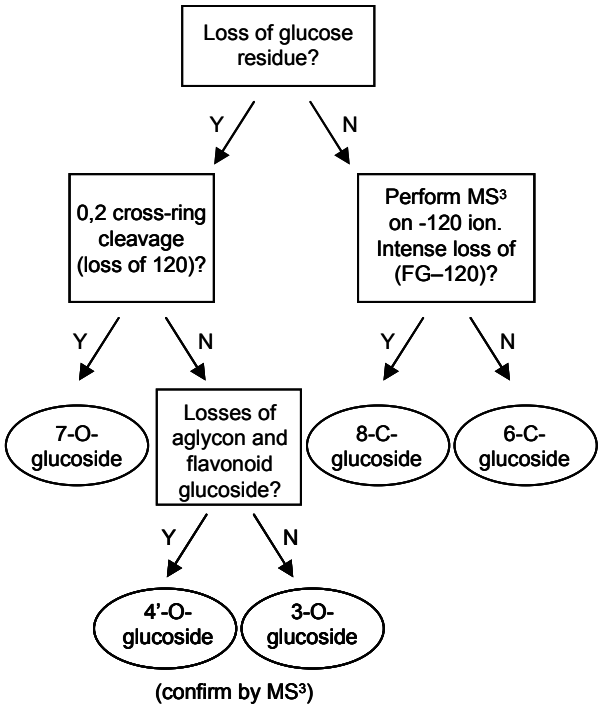
b) MS^3 of –Glc ion

flavonoid glucoside	-Glc	-Agl	$-H_2O$	-FG + adducts
kaempferol 3-O-Glc	20	100	—	—
quercetin 3-O-Glc	12	100	—	—
isorhamnetin 3-O-Glc	17	100	—	—
luteolin 4′-O-Glc	100	80	3	variable
quercetin 4′-O-Glc	12	100	6	variable

c) MS^3 of -120 ion

flavonoid glucoside	-120	-(FG-120)	-60	-[(FG-120) & 60)]	$-H_2O$
apigenin 6-C-Glc	100	3	—	—	3
luteolin 6-C-Glc	100	3	—	—	4
apigenin 8-C-Glc	100	37	6	8	—
luteolin 8-C-Glc	100	21	6	7	—

Relative intensity of each fragment ion is an average of 2 to 4 experiments carried out on different days. Abbreviations: DC-FG is $[2\ Ca(II)\ (FG\text{-}H)_2)\ (FG)_2]^{2+}$ - FG; (FG+H)+ is the protonated flavonoid glucoside; -120 is the 0,2 cross-ring saccharide cleavage, -90 is the 0,3 cross-ring saccharide cleavage, -60 is the 0,4 cross-ring saccharide cleavage, -(FG-120) is the 0,2 cross-ring saccharide cleavage but retaining the cleaved saccharide while losing the aglycon part.



Scheme 3.3. Glycosylation site determination by Ca complexation

molecule and one aglycon unit from the metal complex. However these fragment ions are not always above 5% intensity. For more confident differentiation, a secondary stage of CID was performed to further interrogate the fragment ion stemming from the loss of one glucose moiety. For the 4′-O-glucosyl flavonoid complexes, the resulting MS^3 spectra display a cluster of fragment ions associated with the loss of one flavonoid glucoside molecule, a pathway that is absent or very weak for the 3-O-glucosyl flavonoid complexes (Figure 3.4). The ion stemming from the loss of the flavonoid glucoside is not itself detected, but

several post-dissociation solvent adducts are clearly observed, including the adduction of one or two methanol or water molecules.

The ion labeled as DC-FG ("doubly-charged - flavonoid glycoside") in Table 3.2 appears in the CID spectra of some of the calcium complexes. This ion stems from the parent species $[2\bullet Ca(II)\ (FG\text{-}H)_2\ (FG)_2]^{2+}$. This 4:2 analyte/calcium complex has the same mass/charge ratio as the desired 2:1 complex, so both species are isolated simultaneously in the trap prior to dissociation. Upon activation the 4:2 complex loses one neutral flavonoid

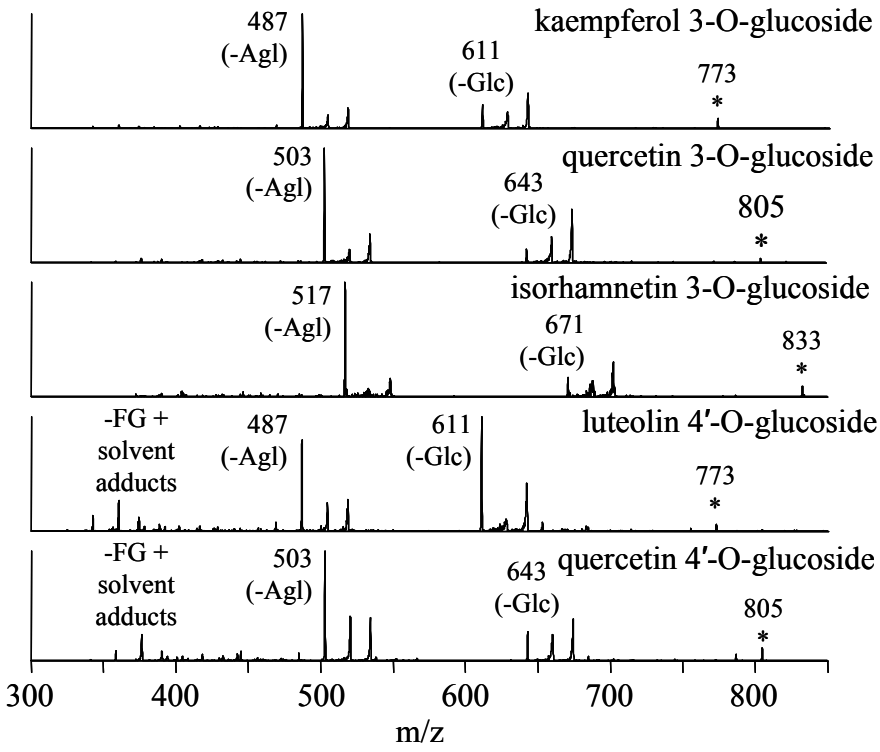


Figure 3.4. MS^3 spectra of Ca(II) complexes involving 3-O- and 4′-O-glucosyl flavonoids. The second stage of CID was performed on the ion stemming from the loss of one glucose residue from $[Ca(II)\ (FG\text{-}H)\ (FG)]^+$.

glucoside molecule to form [2 Ca(II) $(FG\text{-}H)_2$ $(FG)]^{2+}$. Although this dissociation pathway is observed for both 4′-O-glucosyl flavonoids in this study, it is not a useful diagnostic tool as it appears in the spectra of some 3-O- and 7-O-glucosyl flavonoid complexes as well. There may also be a concentration dependence that influences the ratio of 4:2 vs. 2:1 complexes. An excessive amount of the 4:2 complex could mask the fragments from the 2:1 complex, which would complicate identification of the flavonoid glucoside.

The Ca(II) complexes of the 6-C- and 8-C-glucosyl flavonoids display nearly identical CID mass spectra, necessitating the use of MS^3 for differentiation. A further stage of fragmentation of the 0,2 cross-ring cleavage product results in clear spectral differences between these two categories of flavonoid glucosides. The loss of 312 Da (for vitexin and isovitexin) or 328 Da (for orientin and homoorientin) corresponds to the elimination of the remainder of the flavonoid glucoside molecule left after the initial cross-ring cleavage. This fragmentation product, indicated by "-(FG-120)" in Table 3.2, is only significant for the 8-C-glucosyl flavonoids. Another low-intensity diagnostic ion for 8-C-glucosylation is the 0,4 cross-ring cleavage product (-60 Da).

3.3.3 Magnesium Complexes

The [Mg(II) (FG-H) $(FG)]^+$ complexes offer comprehensive identification and differentiation of all five categories of flavonoid glucosides without the need

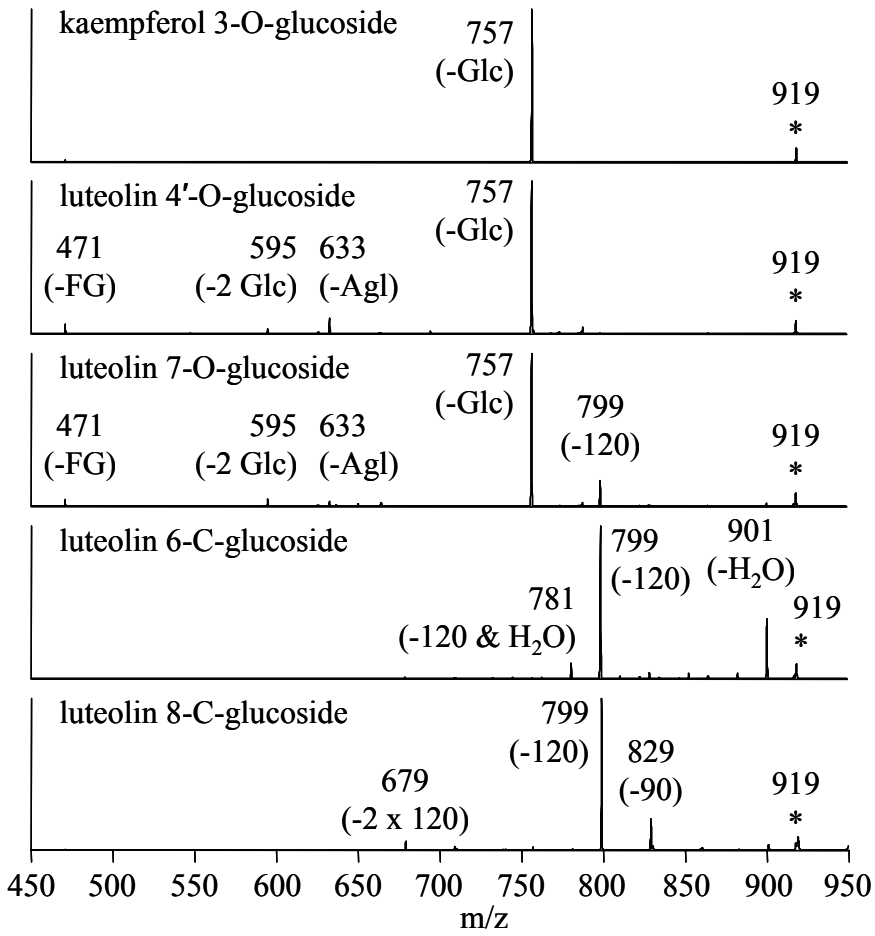


Figure 3.5. CID spectra of $[Mg(II)\ (FG\text{-}H)\ (FG)]^+$ complexes involving isomeric monoglucosyl flavonoids glycosylated at five different positions.

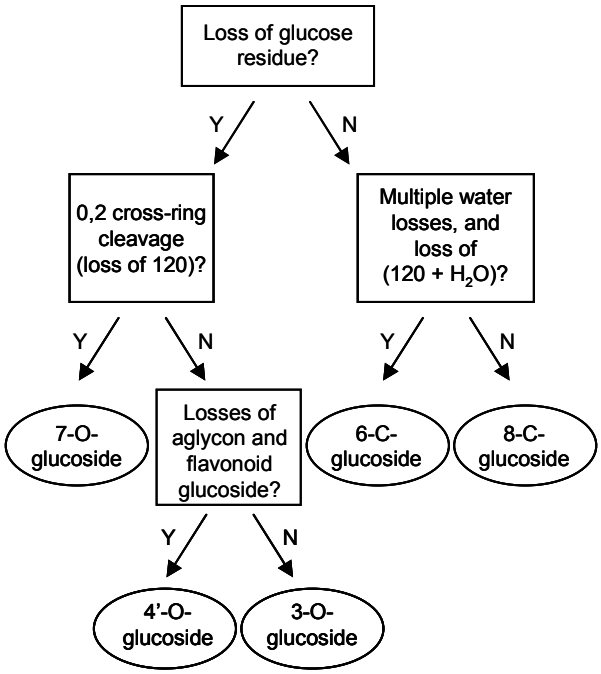
for MS^3 analysis. Figure 3.5 shows the CID spectra of the Mg(II) complexes involving flavonoid glucosides with molecular weight 448. Each analyte is glycosylated at a different site, and the spectra provide an unambiguous means to differentiate these five isomers, and allow determination of the glycosylation site based on diagnostic ions. Table 3.3 lists the major losses from these complexes.

Table 3.3 Selected CID losses from $[Mg(II)\ (FG\text{-}H)\ (FG)]^+$ complexes

flavonoid glucoside	-Glc	-FG	-Agl	-2Glc	-120	$-H_2O$	-2 H_2O	-3 H_2O	-(120 & H_2O	-(2 x120)	-90
kaempferol 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
quercetin 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
isorhamnetin 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
luteolin 4′-O-Glc	100	6	8	3	—	—	—	—	—	—	—
quercetin 4′-O-Glc	100	6	8	—	—	—	—	—	—	—	—
apigenin 7-O-Glc	100	5	5	4	20	5	—	—	—	—	—
naringenin 7-O-Glc	100	3	7	8	8	—	—	—	—	—	—
luteolin 7-O-Glc	100	2	3	5	16	2	—	—	—	—	—
quercetin 7-O-Glc	100	10	5	9	20	5	—	—	—	—	—
apigenin 6-C-Glc	—	—	—	—	100	44	5	3	10	—	4
luteolin 6-C-Glc	—	—	—	—	100	42	5	3	12	—	5
apigenin 8-C-Glc	—	—	—	—	100	4	—	—	—	7	22
luteolin 8-C-Glc	—	—	—	—	100	4	—	—	—	5	18

Relative intensity of each fragment ion is an average of 2 to 4 experiments carried out on different days. Abbreviations: -120 is the 0,2 cross-ring saccharide cleavage, -90 is the 0,3 cross-ring saccharide cleavage.

Loss of the glucose moiety is indicative of O-glucosylation, while the 0,2 cross-ring cleavage of glucose (-120 Da) is the most significant fragmentation pathway for the C-glucosyl flavonoid complexes. Like the calcium complexes, the magnesium complexes offer a means to distinguish the three O-glucosylation sites (Scheme 3.4). Once O-glucosylation has been determined, the 7-O glucosylation site can be confirmed by the presence of the 0,2 cross-ring cleavage pathway. 3-O-glucosyl flavonoid complexes display no significant losses other than that of the glucose moiety, whereas the 4′-O-glucosyl flavonoid complexes also lose an aglycon residue and an entire flavonoid glucoside molecule. These diagnostic ions are sufficiently intense that MS^3 is not necessary for differentiating the 3-O-



Scheme 3.4. Glycosylation site determination by Mg complexation

and 4′-O-glucosyl flavonoids. The CID mass spectra of the magnesium complexes also allow differentiation of the two C-glucosylation positions. Complexes involving 6-C-glucosyl flavonoids undergo a prominent dehydration pathway that involves the loss of up to three water molecules, whereas 8-C-glucosyl flavonoid complexes display only a minor loss of one water molecule. Another indicator of 6-C-glucosylation is the unique fragment resulting from the combination of the 0,2 cross-ring cleavage (-120 Da) and dehydration. Diagnostic ions for 8-C-glucosyl flavonoid complexes include the 0,3 cross-ring cleavage (-90 Da), and the 0,2 cleavage of both glucose moieties.

One concern in relying on the analysis of $[Mg(II)\ (FG\text{-}H)\ (FG)]^+$ complexes for isomer differentiation is the possible isobaric overlap from $[Na\ (FG)_2]^+$ species, which can lead to misidentification due to different fragmentation pathways for the sodium-cationized species. Although sodium adducts are routinely observed in the ESI mass spectra of flavonoid glycosides due to the ubiquitous presence of sodium in the environment, addition of a magnesium salt at the 10^{-5} M level usually results in formation of Mg(II) complexes that significantly outweigh the contribution from sodium adducts for the flavonoid glucosides in this study. Increasing the magnesium concentration solves the problem in cases when there is a significant contribution from the sodium-cationized species.

3.3.4 Manganese Complexes

Mn(II) complexes of the form $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ provide a means to determine glucosylation sites of flavonoid glucosides in a similar manner to magnesium complexation.[40] Table 3.4 summarizes the fragment ions obtained from each complex and shows how this data can be used to determine the glucosylation site. The loss of a glucose moiety, -162 Da, is indicative of O-glucosylation; the complexes of the C-glucosides do not display this loss. The 3-O-glucoside complexes yield no other significant fragmentation products, but the 4′-O-glucoside complexes also display the loss of two glucose moieties, the loss

Table 3.4 Selected CID losses from $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes

flavonoid glucoside	-Glc	-FG	-Agl	-2Glc	-120	$-H_2O$	-2 H_2O	-3 H_2O	-(120 & H_2O	-(2 x120)	-90
kaempferol 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
quercetin 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
isorhamnetin 3-O-Glc	100	—	—	—	—	—	—	—	—	—	—
luteolin 4′-O-Glc	100	8	7	10	—	—	—	—	—	—	—
quercetin 4′-O-Glc	100	5	8	5	—	—	—	—	—	—	—
apigenin 7-O-Glc	100	—	2	8	14	2	—	—	—	—	—
naringenin 7-O-Glc	100	—	3	15	6	—	—	—	—	—	—
luteolin 7-O-Glc	100	2	2	10	13	2	—	—	—	—	—
quercetin 7-O-Glc	100	2	2	12	11	3	—	—	—	—	—
apigenin 6-C-Glc	—	—	—	—	100	40	4	3	15	5	4
luteolin 6-C-Glc	—	—	—	—	100	40	4	3	14	5	4
apigenin 8-C-Glc	—	—	—	—	100	3	—	—	—	6	6
luteolin 8-C-Glc	—	—	—	—	100	2	—	—	—	6	7

Relative intensity of each fragment ion is an average of 2 to 4 experiments carried out on different days. Abbreviations: -120 is the 0,2 cross-ring saccharide cleavage, -90 is the 0,3 cross-ring saccharide cleavage.

of an aglycon unit, and the loss of an intact flavonoid glycoside. It was also noted that the average amount of CID energy required to fragment the 4′-O-glucoside complexes is also higher than that required for the 3-O-glucoside complexes (24.2% versus 18.0%). The 7-O-glucoside complexes are the only ones to display both the loss of a hexose moiety and a 0,2 cross-ring saccharide cleavage (-120 Da). C-glucosylation is indicated by the 0,2 cross-ring cleavage product as the most prominent fragment ion in the CID spectrum. Useful diagnostic ions for differentiating the 6-C- and the 8-C-glucosides include a very abundant dehydration product and the combined loss of 120 Da and a water molecule (both characteristic of 6-C-glucosides). The CID spectra of the 2:1 Mn complexes

involving flavonoid glycosides with molecular weight 448 (spanning five sites of glycosylation) are shown in Figure 3.6. The five-way differentiation of the isomers is immediately apparent upon inspection of the data.

Manganese complexation is superior to the previously described method using magnesium complexation for several reasons. First, the magnesium complexes $[Mg(II)\ (FG\text{-}H)\ (FG)]^+$ suffer from an isobaric overlap with a sodium

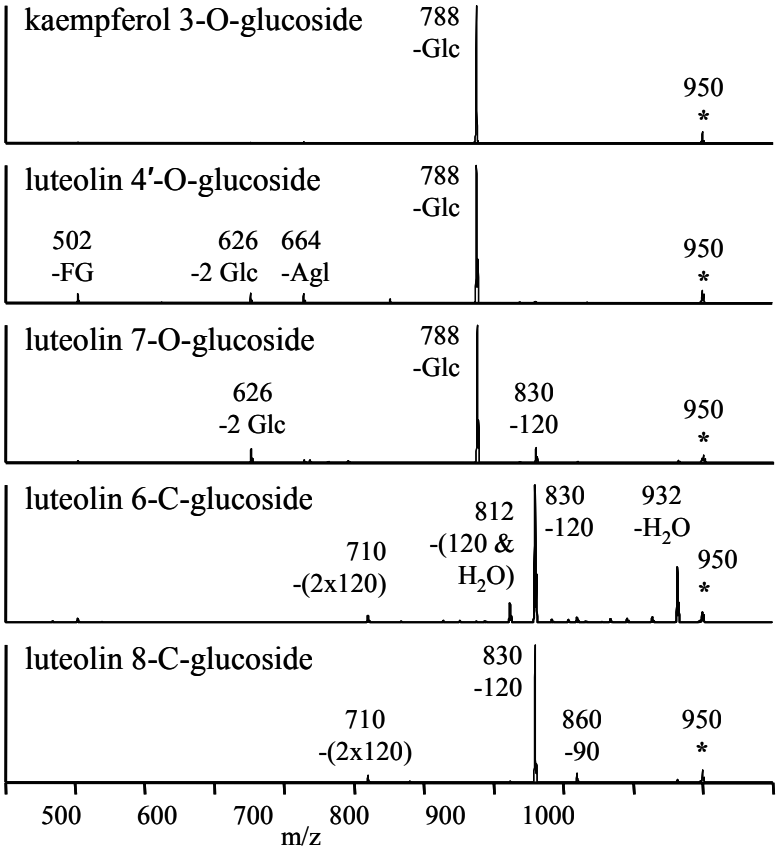


Figure 3.6. CID spectra of $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes of isomeric flavonoid glycosides. Fragment ions are -120 (0,2 cross-ring saccharide cleavage), -90 (0,3 cross-ring saccharide cleavage).

adduct, $[Na\ (FG)_2]^+$ that may complicate spectral interpretation. The Mn(II) complexes do not have this problem. Second, manganese is monoisotopic, leading to a potentially simpler spectrum and greater concentration of the molecular ion intensity into a single peak. Finally, Mn(II) provides the ability to both determine glycosylation sites and to identify the saccharide moiety in the case of 3-O-glycosides (to be discussed in detail in Chapter 4), whereas Mg is not reliable for the latter application.

3.3.5 Cobalt and Nickel Complexes

Tables 3.5 and 3.6 list selected fragment ions observed from the $[Co(II)\ (FG\text{-}H)\ (FG)]^+$ and the $[Ni(II)\ (FG\text{-}H)\ (FG)]^+$ complexes. The O-glycosyl flavonoids and C-glycosyl flavonoids are distinguishable in the same way as with the other metal complexes, discussed earlier. Differentiation of the 6-C-glucosides and the 8-C-glucosides is also possible as complexes involving 6-C-glucosides have a prominent dehydration pathway that involves the loss of up to three water molecules, whereas 8-C-glucoside complexes display only a minor loss of one water molecule. Another indicator of 6-C-glycosylation is the unique fragment resulting from the combination of the 0,2 cross-ring cleavage and the loss of one water molecule. Among the O-glucosyl flavonoids, the 7-O-glucoside complexes are the only ones which display a significant loss of 120 Da. However, there are no unique fragment ions to differentiate the 3-O- and the 4′-O-

Table 3.5 Selected CID losses from $[Co(II)\ (FG\text{-}H)\ (FG)]^+$ complexes

a) MS/MS

flavonoid glucoside	-Glc	-FG	-Agl	-2Glc	-120	$-H_2O$	-2 H_2O	-3 H_2O	-(120 & H_2O	-(2 x120)	-96
kaempferol 3-O-Glc	100	5	8	3	—	—	—	—	—	—	—
quercetin 3-O-Glc	100	4	8	—	—	—	—	—	—	—	—
isorhamnetin 3-O-Glc	100	6	8	6	—	—	—	—	—	—	—
luteolin 4′-O-Glc	100	10	17	3	—	—	—	—	—	—	—
quercetin 4′-O-Glc	100	8	22	3	—	—	—	—	—	—	—
apigenin 7-O-Glc	100	—	3	6	15	2	—	—	—	—	—
naringenin 7-O-Glc	100	2	7	10	10	—	—	—	—	—	—
luteolin 7-O-Glc	100	—	2	6	13	2	—	—	—	—	—
quercetin 7-O-Glc	100	—	3	12	12	3	—	—	—	—	—
apigenin 6-C-Glc	—	5	—	—	100	51	10	8	12	4	6
luteolin 6-C-Glc	—	4	—	—	100	46	10	6	10	4	5
apigenin 8-C-Glc	—	—	—	—	100	4	—	—	—	4	—
luteolin 8-C-Glc	—	—	—	—	100	2	—	—	—	4	—

b) MS^3 of –Glc ion

flavonoid glucoside	-Glc	-Agl	-120	- H_2O
kaempferol 3-O-Glc	95	100	6	6
quercetin 3-O-Glc	78	100	5	4
isorhamnetin 3-O-Glc	100	82	3	2
luteolin 4′-O-Glc	100	33	—	—
quercetin 4′-O-Glc	100	55	3	—

Abbreviations: -120 is the 0,2 cross-ring saccharide cleavage, -96 is the 0,4 cross-ring saccharide cleavage with the loss of two water molecules.

glucosides. Some level of differentiation can be made based on the intensity of fragment ions. MS^3 may also be used to increase confidence in the differentiation, but neither MS/MS nor MS^3 provides fragment ions that are both intense and unique to differentiate these two categories of flavonoids. The combined data from MS/MS and MS^3 should be sufficient to make correct

Table 3.6 Selected CID losses from [Ni(II) (FG-H) (FG)]$^+$ complexes

a) MS/MS

flavonoid glucoside	-Glc	-FG	-Agl	-2Glc	-120	-H_2O	-2 H_2O	-3 H_2O	-(120 & H_2O	-(2 x120)	-96
kaempferol 3-O-Glc	100	5	6	—	—	—	—	—	—	—	—
quercetin 3-O-Glc	100	4	5	—	—	—	—	—	—	—	—
isorhamnetin 3-O-Glc	100	5	—	—	—	—	—	—	—	—	—
luteolin 4′-O-Glc	100	8	16	4	—	—	—	—	—	—	—
quercetin 4′-O-Glc	100	12	36	3	—	—	—	—	—	—	—
apigenin 7-O-Glc	100	—	6	5	15	2	—	—	—	—	—
naringenin 7-O-Glc	100	—	8	12	8	—	—	—	—	—	—
luteolin 7-O-Glc	100	—	5	6	14	3	—	—	—	—	—
quercetin 7-O-Glc	100	2	5	10	10	3	—	—	—	—	—
apigenin 6-C-Glc	—	—	—	—	100	59	18	8	14	4	10
luteolin 6-C-Glc	—	—	—	—	100	50	14	8	13	4	8
apigenin 8-C-Glc	—	—	—	—	100	5	—	—	—	2	—
luteolin 8-C-Glc	—	—	—	—	100	2	—	—	—	2	—

b) MS^3 of –Glc ion

flavonoid glucoside	-Glc	-Agl	-120	- H_2O
kaempferol 3-O-Glc	76	100	9	9
quercetin 3-O-Glc	55	100	5	6
isorhamnetin 3-O-Glc	68	100	2	2
luteolin 4′-O-Glc	100	37	—	—
quercetin 4′-O-Glc	100	79	3	2

Abbreviations: 120 is the 0,2 cross-ring saccharide cleavage, -96 is the 0,4 cross-ring saccharide cleavage with the loss of two water molecules.

identifications, but this is a more complicated approach than using manganese complexation, as described earlier, which only requires MS/MS for glycosylation site determination.

3.3.6 Copper Complexes

The copper complexes display significantly different behavior from the other transition metal complexes. Copper is the only metal ion in this study that shows evidence of reduction to the +1 oxidation state in the full scan spectra. This behavior has been reported previously with electrospray ionization[69-76] and is rationalized by the low reduction potential of Cu(II) due to its ability to achieve a closed-shell d^{10} electronic structure upon gaining an electron. Copper is also unique in its tendency to induce radical losses upon CID of the metal complexes. Losses of the flavonoid glycoside and of the aglycon portion of the flavonoid can occur as neutral or radical losses, or both in the same spectrum. Part of the reason for the divergence from the other transition metal complexes may be due to differences in the favored geometries of complexation (square planar is usual for Cu, whereas tetrahedral or octahedral is common for Co and Ni).[77]

Table 3.7 lists selected fragmentation pathways that occur as a result of CID of the [Cu(II) (FG-H) (FG)]$^+$ complexes. The differentiation between O-glucosyl and C-glucosyl flavonoids is the same as with the other metal complexes. The 6-C-glucoside complexes lose up to three water molecules, with the first loss being particularly intense (65% – 80%). In contrast, the 8-C-glucosides complexes exhibit the loss of one water molecule at an intensity of less than 5%. The difference in the prominence of the dehydration pathway is a clear indicator of 6-C versus 8-C glucosylation.

Table 3.7 Selected CID losses from [Co(II) (FG-H) (FG)]$^+$ complexes

a) MS/MS

flavonoid glucoside	-Glc	-FG	-FG$^\bullet$	-Agl	-Agl$^\bullet$	-2Glc	-H	-120
kaempferol 3-O-Glc	100	12	3	65	—	58	7	—
quercetin 3-O-Glc	100	6	—	51	—	24	—	—
isorhamnetin 3-O-Glc	100	18	3	65	5	39	6	—
luteolin 4′-O-Glc	100	27	3	8	15	5	—	—
quercetin 4′-O-Glc	100	22	11	15	13	—	—	—
apigenin 7-O-Glc	100	—	3	5	—	8	—	4
naringenin 7-O-Glc	100	—	5	12	—	5	6	—
luteolin 7-O-Glc	100	4	73	5	—	4	—	4
quercetin 7-O-Glc	22	15	100	—	—	—	—	—
apigenin 6-C-Glc	—	—	8	—	—	—	7	100
luteolin 6-C-Glc	—	—	19	—	—	—	16	100
apigenin 8-C-Glc	—	—	10	—	—	—	8	100
luteolin 8-C-Glc	—	—	8	—	—	—	5	100

flavonoid glucoside	-H_2O	-2 H_2O	-3 H_2O	-(120 & H_2O	-(120 & 2 H_2O	-(2 x120)	-66	-96
apigenin 6-C-Glc	80	34	22	12	5	3	6	22
luteolin 6-C-Glc	66	28	19	14	5	5	6	19
apigenin 8-C-Glc	3	—	—	—	—	—	—	—
luteolin 8-C-Glc	—	—	—	—	—	—	—	—

b) MS^3 of –Glc ion

flavonoid glucoside	-Glc	-Glc$^\bullet$	-Agl	-Agl$^\bullet$	-120	-H_2O
kaempferol 3-O-Glc	100	—	24	—	4	3
quercetin 3-O-Glc	100	—	21	—	2	2
isorhamnetin 3-O-Glc	—	—	—	—	—	—
luteolin 4′-O-Glc	100	—	76	26	—	—
quercetin 4′-O-Glc	14	—	100	98	—	—
apigenin 7-O-Glc	100	—	—	—	3	—
naringenin 7-O-Glc	100	—	5	2	3	—
luteolin 7-O-Glc	87	2	11	100	—	—
quercetin 7-O-Glc	29	5	21	100	—	39

Abbreviations: -120 is the 0,2 cross-ring saccharide cleavage, -66 is the 2,3 cross-ring saccharide cleavage with the loss of two water molecules, -96 is the 0,4 cross-ring saccharide cleavage with the loss of two water molecules.

However, the 3-O, 4′-O, and 7-O glycosylation positions are not so easily differentiated. The previously-discussed metal complexes allow the easy identification of the 7-O glycosylation site since only 7-O-glucosides undergo both the loss of an intact glucose moiety and a cross-ring cleavage of glucose (-120 Da). With the copper complexes, some 7-O-glucosides do not display the loss of 120 Da, negating this facile identification strategy. It is tempting to use radical cleavages as diagnostic indicators, but there is a problem with relying on these ions. Recent work by Hvattum and Ekeberg has shown that the amount of homolytic versus heterolytic cleavage is dependent on a number of factors, at least for unbound deprotonated flavonoids.[78] These factors include the substituents of the flavonoid aglycon and the amount of collision energy used, in addition to the site of glycosylation. It was shown that the intensity of a saccharide radical loss is dependent on the number of hydroxyl groups on the B ring, correlating positively for 3-O-glycosides but negatively for 7-O-glycosides. The fragmentation pathways observed for copper complexes are different, but similar types of relationships can be found. All four 7-O-glucoside complexes undergo the loss of a flavonoid glycoside radical, but the prominence of this loss correlates with the number of hydroxyl group on the aglycon portion of the molecule. The copper complexes of apigenin 7-O-glucoside and naringenin 7-O-glucoside, both with only two hydroxyl groups on the aglycon subunit, undergo very small amounts of radical loss of a flavonoid. The pathway involving loss of

one glucose moiety is clearly dominant for these complexes. Luteolin 7-O-glucoside has three hydroxyl groups on the aglycon; the result is that fragmentations of the copper complex involving loss of one glucose moiety and the radical loss of one flavonoid are comparable in intensity. Quercetin 7-O-glucoside has four hydroxyl groups on the aglycon, and the dominant pathway for its complex is the radical loss of a flavonoid glycoside. The loss of one glucose moiety is less prominent than for the other 7-O-glycoside complexes. The variability associated with the radical loss therefore affects the appearance of other peaks in the spectrum: it is quite possible that with the addition of another hydroxyl group, the pathway involving the loss of one glucose moiety may no longer be significant. This reduces the utility of that fragmentation pathway as a universal indicator of O-glycosylation. Other examples of the relationship between agylcon structure and radical fragmentation are seen in the MS^3 loss of an aglycon radical for 4′-O- and 7-O-glucoside complexes. In both cases, additional hydroxyl groups on the aglycon greatly increase the amount of radical cleavage, and suppress the intensity of the –Glc fragment ion.

These examples corroborate the observations of Hvattum and Ekeberg, although we are working with copper complexes rather than free flavonoids. Whereas the other metal complexes in this chapter are rather impervious to the structure of the aglycon, the dissociation behavior of the copper complexes is strongly affected by this factor. This may be useful for differentiating individual

isomers, but a universal strategy for determining the glycosylation site should not be strongly affected by other structural features. Therefore copper complexation is not a reliable method for differentiating between the three sites of O-glycosylation.

3.3.7 Cobalt Complexes with an Auxiliary Ligand

The various metal complexation modes discussed above have varying degrees of success in their ability to provide conclusive evidence about the location of the glucose moiety of monoglucosyl flavonoids. However, one common theme is that the 3-O and 4′-O glycosylation sites are the most difficult to differentiate by the metal complexation approach. Even the most generally useful strategies, those involving the complexes [Mg(II) (FG-H) (FG)]$^+$ and [Mn(II) (FG-H) (FG)]$^+$, rely upon low-abundance fragment ions (<10% relative abundance) to make this differentiation. For the calcium, cobalt and nickel complexes, an additional CID step is needed to distinguish these two glycosylation sites, an approach that is not possible on many types of mass spectrometers.

It is worth mentioning another complexation mode that was serendipitously found to provide a simple and obvious differentiation of the 3-O and 4′-O glycosylation sites. The complex in question, [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$, was originally used to identify flavonoid glucuronide metabolites (to

be discussed further in Chapter 6), but was later discovered to also be useful for this application. Unlike the previously discussed work, the working solution consisted of 10 μM flavonoid glycoside, 5 μM $CoBr_2$ and 5 μM auxiliary ligand, in this case 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen). When the relevant complex is dissociated by CID, an obvious difference is seen between the 3-O-glycosyl flavonoid complexes and the 4′-O-glycosyl flavonoid complexes (Figure 3.7). In the case of the former, a prominent fragment ion is observed corresponding to the loss of the saccharide moiety and one auxiliary ligand. This fragment ion is completely absent from the CID spectra of the 4′-O-glycosyl flavonoid complexes. (Some of the fragmentation pathways occur as a mixture of homolytic and heterolytic cleavages, but this does not interfere with the identification process.) Moreover, the unique diagnostic ion is observed not only in the case of 3-O-glucosides, but also when other monosaccharide moieties are present at the 3 position. It does not occur for disaccharides, as evidenced by the examples of kaempferol 7-O-neohesperidoside and quercetin 3-O-rutinoside. This suggests a universal means to identify flavonoids with monosaccharide moieties located at the 3-O position; the precise identity of the saccharide moiety can be determined by other means (see Chapter 4).

The dissociation patterns of the $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ complexes are identical for both 7-O-glucosides and 4′-O-glucosides, so this complexation mode may not be used to differentiate all glycosylation sites, as

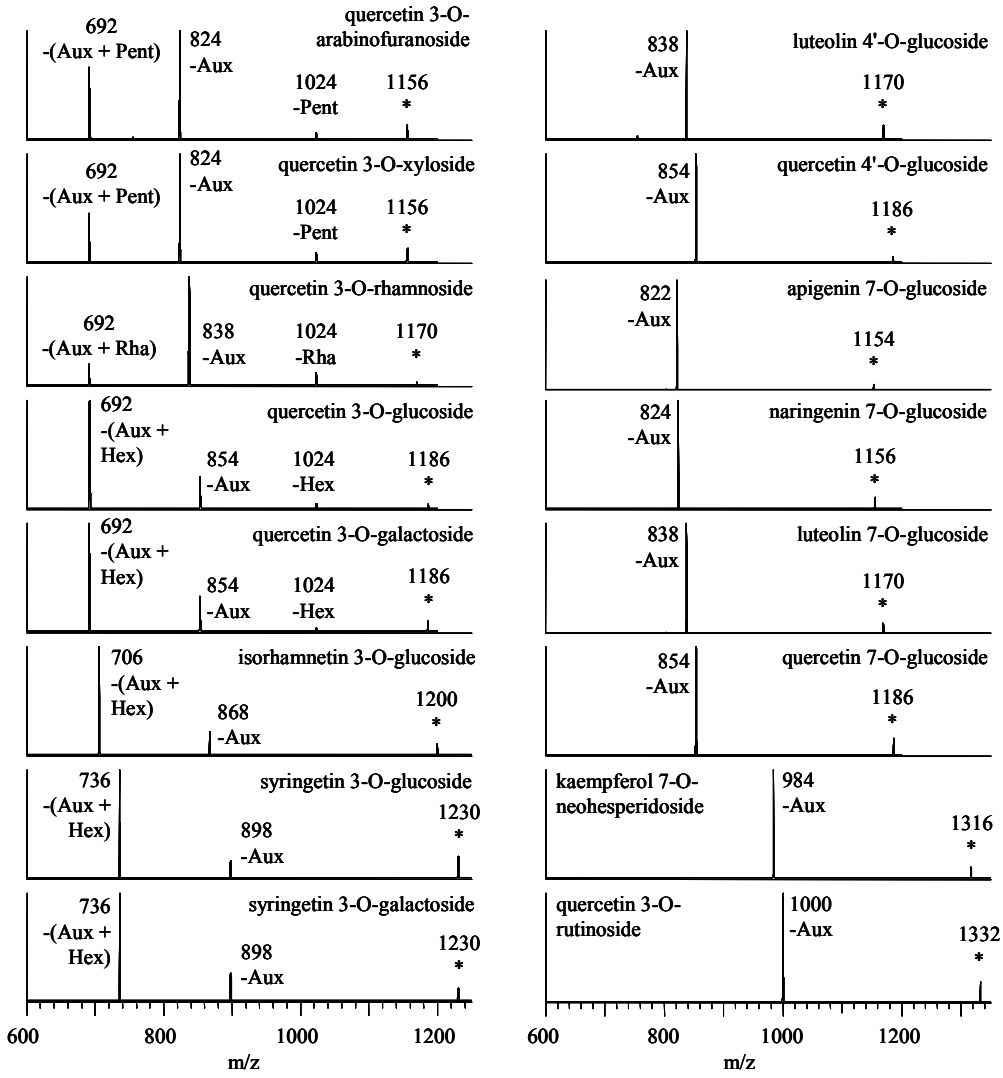


Figure 3.7. CID spectra of [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ complexes of various flavonoid glycosides.

manganese and magnesium complexation could. However, it may be used as a robust means of differentiating the 3-O and 4′-O glycosylation sites in case of ambiguity using other types of metal complexes. This discovery of this complex

invites speculation that diagnostic metal complexation modes may exist providing systematic determination of other structural features of flavonoid glycosides.

3.4 CONCLUSIONS

Dissociation of protonated, deprotonated or sodium-cationized flavonoid monoglucosides does not always provide sufficient diagnostic fragment ions for distinguishing isomers that differ only by their glycosylation site. An alternative approach is to form flavonoid glucoside/metal complexes of the type $[M(II)\ (FG-H)\ (FG)]^+$ which give a broader array of fragments for structural analysis. The dissociation pathways of the complexes are strongly dependent on the glycosylation site, offering a means to determine this important structural feature by mass spectrometry. CID of Mg(II) and Mn(II) complexes resulted in distinctive fragmentation patterns that are indicative of five commonly observed flavonoid glycosylation sites. Ca(II), Co(II) and Ni(II) can also be used to differentiate the glycosylation site if MS^3 is employed. Another type of complex involving the Co(II) ion and an auxiliary ligand, 4,7-diphenyl-1,10-phenanthroline, is especially useful for differentiating 3-O-glycosyl and 4′-O-glycosyl flavonoids, which are only distinguishable by small diagnostic ions using the other methods. All of these metal complexation strategies are effective for hydroxylated and methoxylated flavone, flavonol, and flavanone glucosides; while the cobalt/4,7-dpphen methods can be generalized to monosaccharide

groups other than glucose. Complexes involving Cu(II) were less successful because universal indicators of glycosylation position could not be found for all five glycosylation sites.

3.5 REFERENCES

(1) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
(2) Stobiecki M. *Phytochemistry* **2000**, *54*, 237-256.
(3) Cuyckens F, Ma YL, Pocsfalvi G, Claeys M. *Analusis* **2000**, *28*, 888-895.
(4) Wolfender JL, Maillard M, Marston A, Hostettmann K. *Phytochemical Analysis* **1992**, *3*, 193-214.
(5) Mabry TJ, Ulubelen A. In *Biochemical Applications of Mass Spectrometry, 1st Supplemental Volume*; Waller GR, Dermer OC, Ed.; Wiley: New York, **1980**.
(6) Franski R, Bednarek P, Wojtaszek P, Stobiecki M. *Journal of Mass Spectrometry* **1999**, *34*, 486-495.
(7) Justesen U. *Journal of Chromatography A* **2000**, *902*, 369-379.
(8) Hvattum E. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 655-662.
(9) Maillard MN, Giampaoli P, Cuvelier ME. *Talanta* **1996**, *43*, 339-347.
(10) Becchi M, Fraisse D. *Biomedical and Environmental Mass Spectrometry* **1989**, *18*, 122-130.
(11) Raffaelli A, Moneti G, Mercati V, Toja E. *Journal of Chromatography A* **1997**, *777*, 223-231.
(12) Pietta P, Facino RM, Carini M, Mauri P. *Journal of Chromatography A* **1994**, *661*, 121-126.
(13) Zhang J, Satterfield MB, Brodbelt JS, Britz SJ, Clevidence B, Novotny JA. *Analytical Chemistry* **2003**, *75*, 6401-6407.
(14) Boue SM, Carter-Wientjes CH, Shih BY, Cleveland TE. *Journal of Chromatography A* **2003**, *991*, 61-68.
(15) Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Bastida J, Viladomat F, Codina C. *Journal of Chromatography A* **2003**, *1008*, 57-72.
(16) Sanchez-Rabaneda F, Jauregui O, Casals I, Andres-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventos RM. *Journal of Mass Spectrometry* **2003**, *38*, 35-42.
(17) Llorach R, Gil-Izquierdo A, Ferreres F, Tomas-Barberan FA. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 3895-3899.
(18) Edenharder R, Keller G, Platt KL, Unger KK. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2767-2773.

(19) Satterfield M, Black DM, Brodbelt JS. *Journal of Chromatography B* **2001**, *759*, 33-41.
(20) Nielsen SE, Freese R, Cornett C, Dragsted LO. *Analytical Chemistry* **2000**, *72*, 1503-1509.
(21) Mauri PL, Iemoli L, Gardana C, Riso P, Simonetti P, Porrini M, Pietta PG. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 924-931.
(22) Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(23) Kuhn F, Oehme M, Romero F, Abou-Mansour E, Tabacchi R. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.
(24) Hughes RJ, Croley TR, Metcalfe CD, March RE. *International Journal of Mass Spectrometry* **2001**, *210/211*, 371-385.
(25) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
(26) Zhang J, Brodbelt JS. *Journal of Mass Spectrometry* **2003**, *38*, 555-572.
(27) Ma YL, Vedernikova I, Van den Heuvel H, Claeys M. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 136-144.
(28) Cuyckens F, Rozenberg R, de Hoffmann E, Claeys M. *Journal of Mass Spectrometry* **2001**, *36*, 1203-1210.
(29) Ma YL, Cuyckens F, Van den Heuvel H, Claeys M. *Phytochemical Analysis* **2001**, *12*, 159-165.
(30) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(31) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(32) Cuyckens F, Shahat AA, Pieters L, Claeys M. *Journal of Mass Spectrometry* **2002**, *37*, 1272-1279.
(33) Ma YL, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 1932-1942.
(34) Justesen U. *Journal of Mass Spectrometry* **2001**, *36*, 169-178.
(35) Prasain JK, Patel R, Kirk M, Wilson L, Botting N, Darley-Usmar VM, Barnes S. *Journal of Mass Spectrometry* **2003**, *38*, 764-771.
(36) Zhang J, Brodbelt J. *Journal of the American Chemical Society* **2004**, *126*, 5906-5919.
(37) Li QM, Van den Heuvel H, Dillen L, Claeys M. *Biological Mass Spectrometry* **1992**, *21*, 213-221.
(38) Waridel P, Wolfender JL, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Journal of Chromatography A* **2001**, *926*, 29-41.
(39) Bakhtiar A, Gleye J, Moulis C, Fouraste I. *Phytochemical Analysis* **1994**, *5*, 86-89.

(40) Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
(41) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2005**, *40*, 364-372.
(42) Alvarez EJ, Vartanian VH, Brodbelt JS. *Analytical Chemistry* **1997**, *69*, 1147-1155.
(43) Alvarez EJ, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 463-472.
(44) Shen J, Brodbelt JS. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 1381-1389.
(45) Smith G, Leary JA. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 953-957.
(46) Gaucher SP, Leary JA. *Analytical Chemistry* **1998**, *70*, 3009-3014.
(47) Gaucher SP, Leary JA. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 269-272.
(48) Gaucher SP, Leary JA. *International Journal of Mass Spectrometry* **2000**, *197*, 139-148.
(49) Konig S, Leary JA. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1125-1134.
(50) Desaire H, Leary JA. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 1086-1094.
(51) Desaire H, Beyer MK, Leary JA. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 517-527.
(52) Desaire H, Leary JA. *International Journal of Mass Spectrometry* **2001**, *209*, 171-184.
(53) Basso E, Chilin A, Guiotto A, Traldi P. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 2781-2787.
(54) Ho YP, Huang PC, Deng KH. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 114-121.
(55) Asam MR, Glish GL. *Journal of the American Society for Mass Spectrometry* **1997**, *8*, 987-995.
(56) Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2000**, *35*, 566-571.
(57) Vaisar T, Gatlin CL, Rao RD, Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2001**, *36*, 306-316.
(58) Carlesso V, Fournier F, Tabet JC. *European Journal of Mass Spectrometry* **2000**, *6*, 421-428.
(59) Satterfield M, Brodbelt JS. *Analytical Chemistry* **2000**, *72*, 5898-5906.
(60) Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.
(61) McClellan JE, Murphy JP, Mulholland JJ, Yost RA. *Analytical Chemistry* **2002**, *74*, 402-412.

(62) Cuyckens F, Claeys M. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 2341-2348.
(63) Rauha JP, Vuorela H, Kostiainen R. *Journal of Mass Spectrometry* **2001**, *36*, 1269-1280.
(64) de Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2003**, *984*, 45-58.
(65) Moridani MY, Pourahmad J, Bui H, Siraki A, O'Brien PJ. *Free Radical Biology and Medicine* **2003**, *34*, 243-253.
(66) Weber G. *Chromatographia* **1988**, *26*, 133-138.
(67) de Souza RFV, Sussuchi EM, De Giovani WF. *Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry* **2003**, *33*, 1125-1144.
(68) Soczynska-Kordala M, Bakowska A, Oszmianski J, Gabrielska J. *Cellular & Molecular Biology Letters* **2001**, *6*, 277-281.
(69) Fernandez MT, Mira ML, Florencio MH, Jennings KR. *Journal of Inorganic Biochemistry* **2002**, *92*, 105-111.
(70) Shen J, Brodbelt J. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 126-135.
(71) Lavanant H, Virelizier H, Hoppilliard Y. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1217-1221.
(72) Chaparro AL, Vachet RW. *Journal of Mass Spectrometry* **2003**, *38*, 333-342.
(73) Gatlin CL, Turecek F, Vaisar T. *Analytical Chemistry* **1994**, *66*, 3950-3958.
(74) Blades AT, Jayaweera P, Ikonomou MG, Kebarle P. *International Journal of Mass Spectrometry and Ion Processes* **1990**, *102*, 251-267.
(75) Vaisar T, Gatlin CL, Turecek F. *International Journal of Mass Spectrometry and Ion Processes* **1997**, *162*, 77-87.
(76) Xu YD, Zhang X, Yergey AL. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 25-29.
(77) Cotton FA, Wilkinson G. *Advanced Inorganic Chemistry: A Comprehensive Text*; Interscience Publishers: New York, **1966**.
(78) Hvattum E, Ekeberg D. *Journal of Mass Spectrometry* **2003**, *38*, 43-49.

Chapter 4: Differentiation of Isomeric Saccharides in Monoglycosyl Flavonoids Using Manganese Complexation and Tandem Mass Spectrometry

4.1 Introduction

The previous chapter covered glycosylation site determination of monoglycosyl flavonoids, particularly flavonoid glucosides. This chapter instead focuses on identifying the saccharide moieties of monoglycosyl flavonoids. This is a particularly difficult problem because many isomeric saccharides are known to occur in natural flavonoid conjugates. Typically either NMR spectrometry or comparison to standards is needed to identify these saccharide moieties. The UV spectroscopy method discussed in Section 1.3 can not identify the saccharide moieties of flavonoid glycosides, though it is effective for assigning the glycosylation site.[1,2] Due to the difficulty in characterizing flavonoid glycosides, some researchers opt to remove the saccharide moieties via hydrolysis prior to analysis and thus identify only the aglycon portion of molecules in their extracts and samples.[3-8] As the saccharide moieties play an important though not well-understood role in determining bioactivity, such an approach results in the loss of critical information.

A few tandem mass spectrometry methods have been reported for differentiating isomeric saccharides.[9-17] There are also several reports of systematic differentiation between flavonoid rutinosides and neohesperidosides,

such as hesperidin and neohesperidin, shown in Figure 1.3. This may be done using metal complexation[18-25] or without it.[26-31] Other than these applications, very little has been published regarding systematic saccharide identification of flavonoid glycosides using mass spectrometry. Cuyckens and Claeys published the only available mass spectrometric method for identifying isomeric monosaccharide moieties (i.e. glucose vs. galactose) of flavonoid glycosides,[32] but their method requires isolation and an overnight derivatization of the analytes prior to analysis. This chapter covers an alternative means to differentiate the isomeric saccharide moieties of monoglycosyl flavonols, flavones, and flavanones. The approach involves the use of manganese complexation to yield saccharide-specific fragmentation. Unlike the method of Cuyckens and Claeys, the metal derivatization occurs quickly and does not require prior isolation of the flavonoid glycoside analytes.

4.2 EXPERIMENTAL

Quercetin 3-O-glucoside (isoquercitrin), quercetin 3-O-galactoside (hyperoside), syringetin 3-O-glucoside and syringetin 3-O-galactoside were purchased from Extrasynthèse (Genay, France). Kaempferol 3-O-glucoside (astragalin), isorhamnetin 3-O-glucoside and quercetin 3-O-rhamnoside (quercitrin) were purchased from Indofine (Somerville, NJ). Quercetin 3-O-arabinofuranoside (avicularin) and quercetin 3-O-xyloside (reynoutrin) were

purchased from Apin (Abingdon, UK). Manganese (II) chloride was purchased from Aldrich (Milwaukee, WI). All materials were used without further purification.

All experiments were performed using an LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA) with an electrospray ionization (ESI) source. For direct infusion experiments, the sample introduction rate was 5 μL/min and the ion injection time was 10 msec for full scans and 50 msec for collision-induced dissociation (CID) experiments. 100 microscans were averaged for each spectrum. The metal complexes were analyzed in positive ion mode using the following spray conditions: spray voltage, +5 kV; sheath gas flow rate, 5 arbitrary units; no auxiliary gas; heated capillary temperature, 200 °C; capillary voltage, 20 V; tube lens offset, 20 V. Analyte solutions were made in methanol, with the flavonoid standard and metal salt each added at 1.0×10^{-5} M. The ion abundances from the CID experiments are reported relative to the most abundant ion in the spectrum, which is designated as 100%. When collecting CID data, the collision energy was chosen such that the precursor ion was reduced to 5-10% relative abundance. Collision energies are reported as a percentage of the maximum 5 V_{p-p}, normalized for the m/z of the parent.[33] Isolation windows of 4-6 m/z were used because many of the metal complexes require a wide window in order to yield a stable fragmentation spectrum. Several different types of non-

covalent complexes have been reported to require wider than average windows for effective isolation[34-36] due to their fragility.[37]

4.3 Results and Discussion

4.3.1 Initial Results

The goal of this project was to develop a simple MS method for differentiating isomeric monoglycosyl flavonoids that differ only in the identity of the saccharide moiety. The first indication that this might be possible came from examining the fragmentation pathways of metal complexes involving a flavonoid rhamnoside (quercitrin). There was interest in determining whether a flavonoid rhamnoside complex would dissociate by pathways similar to those of the flavonoid glucosides. The fragmentation pathways from quercitrin/metal complexes were thus compared to the complexes of the flavonoid glucosides.

There were a number of differences in the spectra of the complexes involving quercitrin. One example is the MS/MS fragmentation of 2:1 quercitrin/Ni(II) complexes, in which the loss of one aglycon unit is a more significant fragmentation pathway than for complexes of the 3-O-glucosyl flavonoids (Figure 4.1). Comparison of the MS^3 results, where the parent ion is the 2:1 flavonoid glycoside/Ni(II) complex after the loss of one saccharide moiety, reveals even more striking differences (Figure 4.2). The dehydration pathway is much more significant for the quercitrin complex, which shows

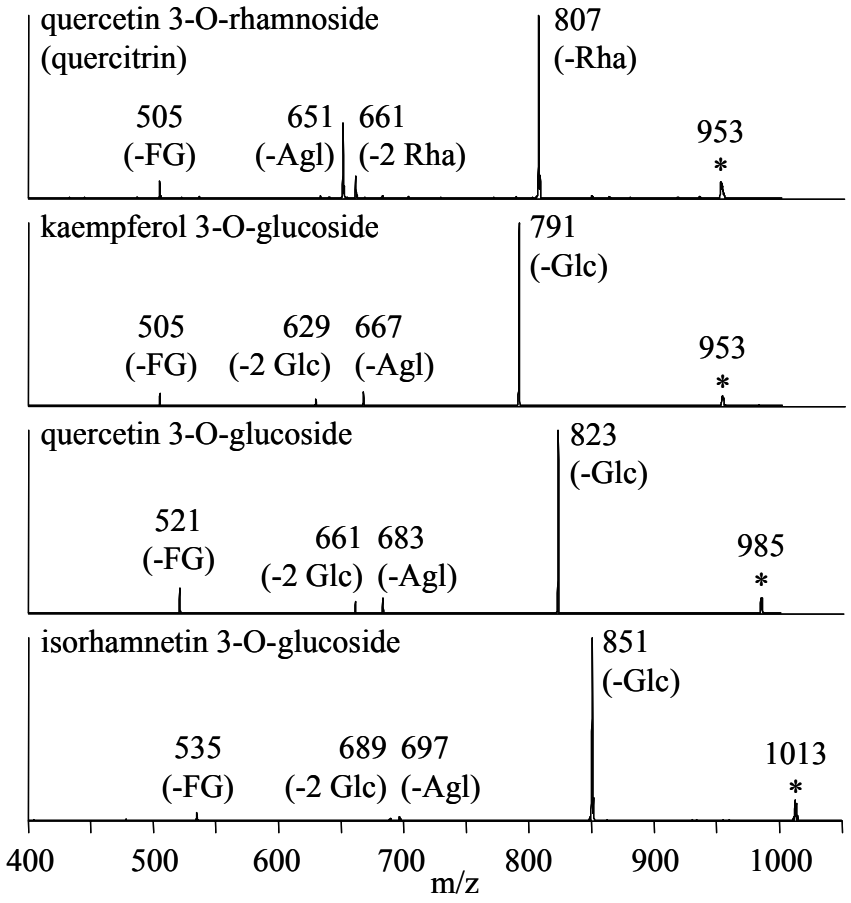


Figure 4.1. MS/MS spectra of [Ni/(II) (FG-H) (FG)]$^+$ complexes of isomeric flavonoid glycosides.

intense losses of one and two water molecules, compared to a minor loss of just one water molecule for the analogous glucoside complexes. The cross-ring cleavages are also more significant and more varied. (Because rhamnose is a deoxyhexose, the mass of the cleaved portion of the saccharide is 16 Da less than it would be for glucose.) The 0,2 cross-ring saccharide cleavage (m/z 703) is the most dominant fragment for the quercitrin/Ni(II) complex, but the corresponding

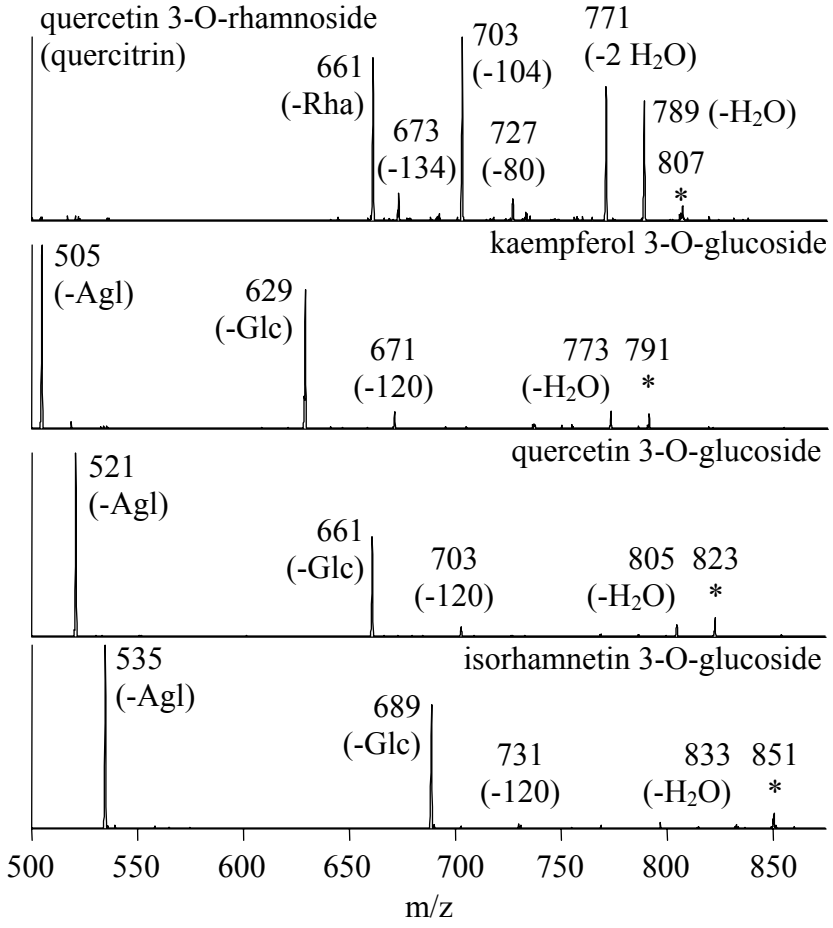


Figure 4.2. MS^3 spectra of $[Ni/(II)\ (FG\text{-}H)\ (FG)]^+$ complexes of isomeric flavonoid glycosides.

cleavage (-120 Da) is minor for the flavonoid glucoside complexes. The quercitrin complex also displays a 0,1 cleavage (-134 Da) and a 0,4 cleavage followed by loss of two water molecules (-80 Da), neither of which is observed for the flavonoid glucoside complexes. Furthermore, the quercitrin complex does not undergo a significant loss of an aglycon unit, which is the dominant loss for the three flavonoid glucoside complexes. These results show that the identity of

the saccharide can play an important role in determining the fragmentation pathways of metal complexes.

Flavonoid rhamnosides are easily distinguishable from other types of monoglycosyl flavonoids because rhamnose is the only deoxyhexose that commonly forms conjugates with flavonoids. The distinctive mass loss due to cleavage of the saccharide moiety (-146 Da) is sufficient to identify flavonoid rhamnosides. In contrast, several hexoses and pentoses are known to form conjugates with flavonoids, such that losses due to intact saccharide cleavage are not sufficient to identify the monosaccharide. While glucose is the most typical hexose found in naturally-occurring flavonoid glycosides, galactose is also common. These two monosaccharides are diastereomers, differing only in their stereochemistry (Figure 4.3). Furthermore, several isomeric pentoses, including arabinose and xylose, are observed in naturally-occurring flavonoid glycosides.

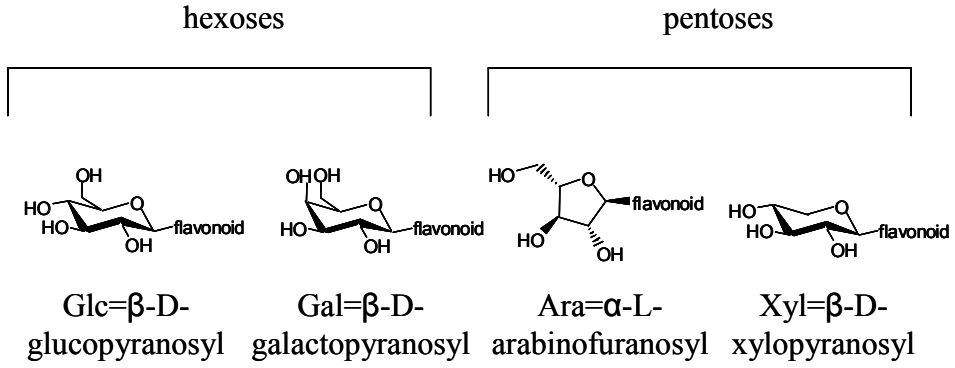


Figure 4.3. Structures of isomeric monosaccharide moieties, with points of attachment to the flavonoid indicated.

Tandem mass spectrometry using protonated, deprotonated and sodium-cationized flavonoid glycosides does not provide a sufficient means for differentiating the isomeric saccharide moieties of these compounds. But the results obtained from metal complexation involving quercitrin were promising in terms of developing a similar strategy to identify isomeric monosaccharides conjugated to flavonoids. Thus, a wide variety of metals, including Mg, Ca, Sr, Ba, Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), K, Ag, and Al was evaluated for this purpose in the present study. ESI-MS analysis of solutions containing a flavonoid glycoside and a metal(II) salt resulted in detection of 1:1 complexes, $[M(II)\ (FG - H)]^+$, and 2:1 complexes, $[M(II)\ (FG - H)\ (FG)]^+$. The ion abundance from these metal complexes was typically on the same order as the protonated flavonoid glycoside, $(FG+H)^+$, with the 2:1 complex generally more intense than the 1:1 complex. The fragmentation patterns of both the 1:1 and 2:1 flavonoid glycoside/metal complexes were evaluated, and MS^3 fragmentation was performed on key diagnostic fragment ions. Some degree of isomer differentiation was possible using the MS/MS spectra of several of the 1:1 complexes; however the fragmentation patterns were always very complicated and subject to post-dissociation solvent adduction, and in many cases differentiation was based only on *relative intensities* of fragment ions rather than on the presence or absence of *unique diagnostic* fragment ions. In contrast, the

MS/MS spectra of the 2:1 complexes were much simpler, but usually did not provide sufficient differentiation of isomers.

4.3.2. Manganese Complexation

The best differentiation of the flavonoid glucoside/galactoside pairs comes from MS^3 experiments using the Mn(II) complexes. Several species are observed in the full scans (Figure 4.4), including $(FG+H)^+$ and $[Mn(II)\ (FG-H)]^+$, but typically the most abundant species under the working conditions is the 2:1 complex, $[Mn(II)\ (FG)\ (FG-H)]^+$. When the 2:1 complexes of the 3-O-hexosides are subjected to CID, the only fragment ion is the result of the loss of one hexose moiety, -162 Da (data not shown). However, performing a second stage of CID

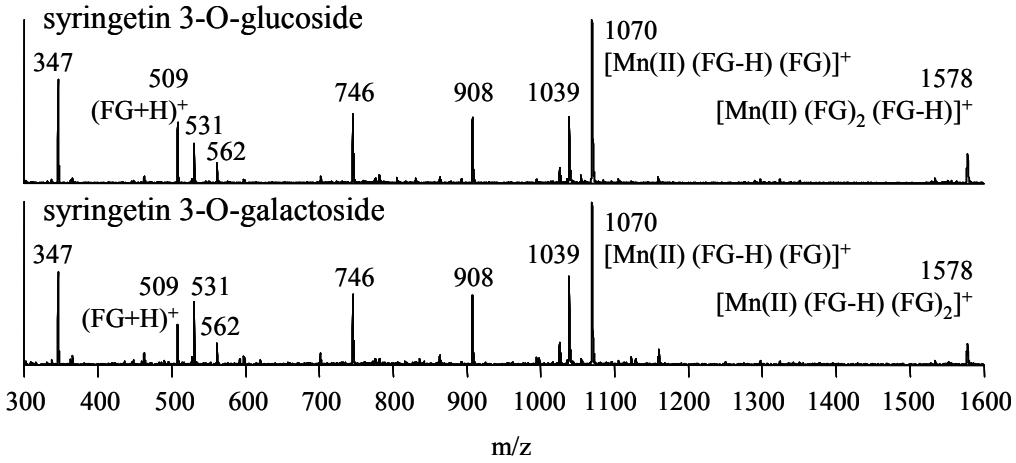


Figure 4.4. Full scan negative ion mode spectra of selected flavonoid glycosides (FG) with Mn(II). Ions are identified as 347: $(FG+H)^+$ – Hex; 531: $(FG+Na)^+$; 562: $[Mn(II)\ (FG-H)]^+$; 746: $[Mn(II)\ (FG-H)\ (FG)]^+$ – 2 Hex; 908: $[Mn(II)\ (FG-H)\ (FG)]^+$ – Hex; 1039: $(2\ FG+Na)^+$.

on this key primary fragment ion leads to a clear differentiation of the 3-O-glucosides and –galactosides (Figure 4.5). All of the complexes exhibit losses of the second hexose moiety and of one aglycon unit, but the two flavonoid galactoside complexes display the additional loss of an aglycon plus 102 Da, providing a means to distinguish these compounds from the corresponding flavonoid glucosides. The loss of 102 Da from saccharide/metal complexes was

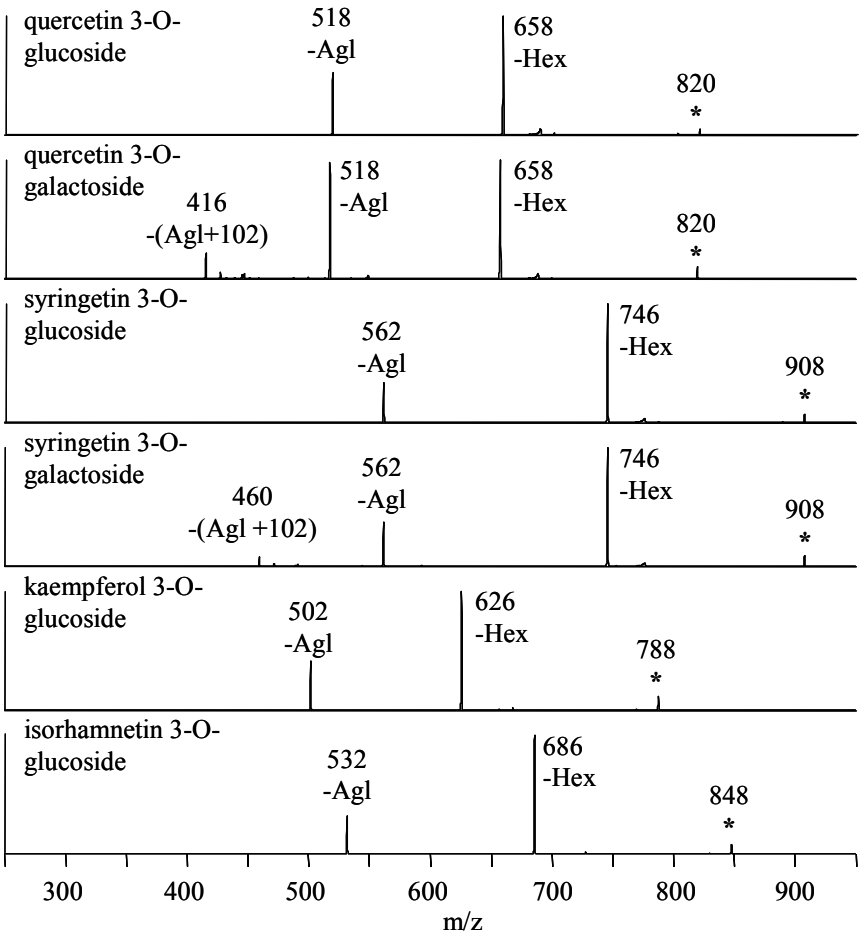
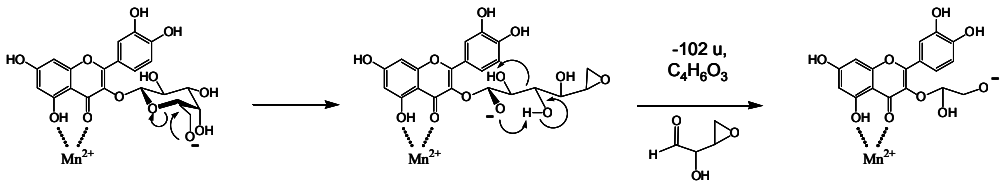


Figure 4.5. MS^3 spectra of $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes of 3-O-hexosides following the loss of one hexose moiety. The CID energy was 22-23% in all cases.

reported by Gaucher and Leary in a study to differentiate hexose sugars using a Zn(II)-dien complex.[9] They performed isotopic labeling studies indicating that sugar carbons 3 through 6 were lost. Based on this information, they proposed a mechanism for this fragmentation which we have adapted for the present case (Scheme 4.1). Starting with the 2:1 complex, one galactoside moiety is lost in the first stage of dissociation, followed by the aglycon in the second stage, in effect leaving behind a 1:1 complex. The additional loss of 102 Da is attributed to a rearrangement/fragmentation of the remaining galactose moiety, resulting in the elimination of $C_4H_6O_3$. The flavonoid glucoside complexes do not display this fragmentation pathway even up to 50% CID energy, presumably because of conformational differences which disfavor this process.

It is interesting that the $[Mn(II)\ (FG)\ (FG\text{-}H)]^+$ complexes of flavonoid 3-O-galactosides fragment identically to the analogous complexes of flavonoid 3-O-glucosides via MS/MS, yet the difference is revealed in the MS^3 experiment. The significance of this result is that the glycosylation site of flavonoid 3-O-glucosides and 3-O-galactosides may be determined in the same manner.



Scheme 4.1. Proposed mechanism for the loss of 102 Da from Mn complexes of flavonoid galactosides (hyperoside shown as example).

Conveniently, the same complex is able to differentiate these isomeric saccharide moieties. MS/MS of the analogous complex involving quercetin 3-O-arabinofuranoside yielded a different fragmentation pattern. Thus such complexes are not able to universally indicate 3-O-glycosylation. The only metal complex that has thus far shown a saccharide-independent glycosylation site signature is $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ for 3-O-glycosides (see Section 3.3.7), and then only for monosaccharide derivatives.

None of the 2:1 flavonoid glycoside/metal complexes provides a satisfactory differentiation of quercetin 3-O-arabinofuranoside and quercetin 3-O-xyloside. Instead, differentiation is achieved based on CID of the 3:1 Mn complexes, $[Mn(II)\ (FG\text{-}H)\ (FG)_2]^+$ (Figure 4.6). Below 20% CID energy, the arabinoside complex gives only one signficant product ion stemming from the loss of one flavonoid glycoside. However the xyloside complex also yields an

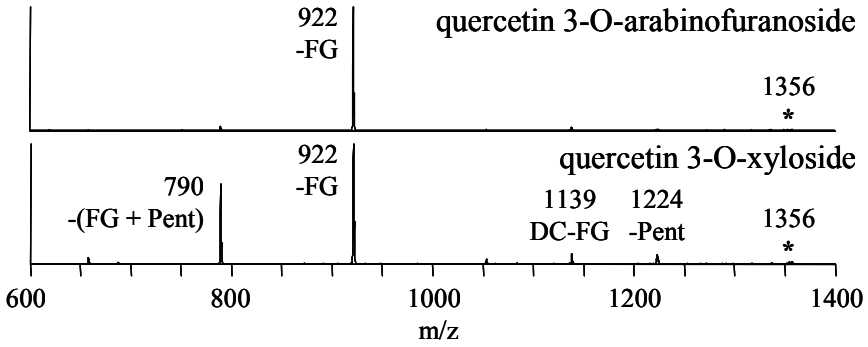


Figure 4.6. MS/MS spectra of $[Mn(II)\ (FG\text{-}H)\ (FG)_2]^+$ complexes of 3-O-pentosides. DC-FG refers to $([2\ Mn(II)\ (FG\text{-}H)_2\ (FG)_4]^{2+}$ - FG).

abundant fragment ion corresponding to the loss of a flavonoid glycoside plus a pentose moiety (m/z 790), in addition to some lower abundance fragments. Using a CID energy around 17% provides the best differentiation of the two isomers.

4.4 CONCLUSIONS

Performing CID on complexes of the form $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ and $[Mn(II)\ (FG\text{-}H)\ (FG)_2]^+$ provides information on the glycosylation site and saccharide identity of these compounds based on unique and consistent fragmentation patterns. There is a robust differentiation of glucose and galactose moieties, at least at the 3-O position, as determined by the similarity of fragment ions for glycosides based on two different flavonoids, quercetin and syringetin. This type of differentiation will be shown in Chapter 5 to hold for derivatives of kaempferol and isorhamnetin derivatives as well. The differentiation of xylose and arabinose has only been demonstrated on quercetin derivatives; therefore it cannot be concluded that the same strategy will work on other compounds. Additionally, arabinose is known to form conjugates in either its 5-membered ring form (furanoside) or its 6-membered ring form (pyranoside). No standards are available to search for contrasting fragmentation of arabinofuranosides and arabinopyranosides, so currently this differentiation is not currently possible using the metal complexation approach.

4.5 REFERENCES

(1) Markham KR. *Techniques of Flavonoid Identification*; Academic Press: London; New York, **1982**.
(2) de Rijke E, Out P, Niessen WMA, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2006**, *1112*, 31-63.
(3) Marotti M, Piccaglia R. *Journal of Food Science* **2002**, *67*, 1229-1232.
(4) Patil BS, Pike LM, Yoo KS. *Journal of the American Society for Horticultural Science* **1995**, *120*, 909-913.
(5) Lee JH, Lee JY, Kim KN, Kim HS. *Food Science and Biotechnology* **2003**, *12*, 415-418.
(6) Tokusoglu O, Unal MK, Yildirim Z. *Acta Chromatographica* **2003**, *13*, 196-207.
(7) Justesen U, Knuthsen P. *Food Chemistry* **2001**, *73*, 245-250.
(8) Chen H, Zuo YG, Deng YW. *Journal of Chromatography A* **2001**, *913*, 387-395.
(9) Gaucher SP, Leary JA. *Analytical Chemistry* **1998**, *70*, 3009-3014.
(10) Gaucher SP, Leary JA. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 269-272.
(11) Gaucher SP, Leary JA. *International Journal of Mass Spectrometry* **2000**, *197*, 139-148.
(12) Desaire H, Beyer MK, Leary JA. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 517-527.
(13) Desaire H, Leary JA. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 1086-1094.
(14) Desaire H, Leary JA. *International Journal of Mass Spectrometry* **2001**, *209*, 171-184.
(15) Carlesso V, Fournier F, Tabet JC. *European Journal of Mass Spectrometry* **2000**, *6*, 421-428.
(16) Smith G, Leary JA. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 953-957.
(17) Konig S, Leary JA. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1125-1134.
(18) Zhang JM, Wang JM, Brodbelt JS. *Journal of Mass Spectrometry* **2005**, *40*, 350-363.
(19) Zhang JM, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1761-1770.
(20) Zhang JM, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 139-151.
(21) Zhang J, Brodbelt J. *Journal of the American Chemical Society* **2004**, *126*, 5906-5919.

(22) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(23) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(24) Pikulski M, Aguilar A, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2007**, *18*, 422-431.
(25) Pikulski M, Wilson JJ, Aguilar A, Brodbelt JS. *Analytical Chemistry* **2006**, *78*, 8512-8517.
(26) Ma YL, Cuyckens F, Van den Heuvel H, Claeys M. *Phytochemical Analysis* **2001**, *12*, 159-165.
(27) Cuyckens F, Rozenberg R, de Hoffmann E, Claeys M. *Journal of Mass Spectrometry* **2001**, *36*, 1203-1210.
(28) Li QM, Van den Heuvel H, Dillen L, Claeys M. *Biological Mass Spectrometry* **1992**, *21*, 213-221.
(29) Cuyckens F, Ma YL, Pocsfalvi G, Claeys M. *Analusis* **2000**, *28*, 888-895.
(30) Li QM, Claeys M. *Biological Mass Spectrometry* **1994**, *23*, 406-416.
(31) Careri M, Elviri L, Mangia A. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 2399-2405.
(32) Cuyckens F, Shahat AA, Pieters L, Claeys M. *Journal of Mass Spectrometry* **2002**, *37*, 1272-1279.
(33) Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.
(34) Vrkic AK, O'Hair RAJ. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 715-724.
(35) Vaisar T, Gatlin CL, Turecek F. *International Journal of Mass Spectrometry and Ion Processes* **1997**, *162*, 77-87.
(36) Jellen EE, Chappell AM, Ryzhov V. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 1799-1804.
(37) McClellan JE, Murphy JP, Mulholland JJ, Yost RA. *Analytical Chemistry* **2002**, *74*, 402-412.

Chapter 5: Liquid Chromatography-Tandem Mass Spectrometry Analysis of Monoglycosyl Flavonoids in Food and Plant Extracts Using Postcolumn Manganese Complexation

5.1 INTRODUCTION

The previous two chapters detail how metal complexation methods in conjunction with mass spectrometry can be used to provide conclusive identifications of monoglycosyl flavonoids. However, these experiments involved commercially-available analytical standards, which were pre-purified and pre-characterized. Any useful method that purports to identify flavonoid glycosides *de novo* must be tested on real samples. Furthermore, it is extremely difficult and time-consuming to purify each component of a complex mixture prior to qualitative analysis. Therefore, the next goal was to adapt the previously-described metal complexation methods to LC-MS analysis in order to eliminate the need to purify individual compounds, and to test the effectiveness of the analytical methods against real food and botanical extracts. By combining purification and identification into a single step, the methods described herein provide an efficient and effective means to obtain qualitative information about monoglycosyl flavonoids in complex mixtures.

First, an assessment of the sample requirements for the LC-MS technique is given. The results of three applications are then presented. The flavonoid contents of apple peel and red onions have already been studied, so these analytes

provide a means to compare the information obtained by the new technique of LC-MS combined with metal complexation to more established (and more time-consuming) techniques. Apple peel is known to contain flavonoid glycosides with many different saccharide groups,[1-5] so it provides a good opportunity to test the saccharide identification technique described in Chapter 4 on a real sample. Onions, on the other hand, produce flavonoid glycosides with varying O-glycosylation sites,[6-10] which makes it ideal for testing methods for determining the position of saccharide groups as discussed in Chapter 3. After the analyses were completed, literature reports on the contents of these foods were used to gauge the success of identifying the various flavonoid glycosides in the extracts.

The third application involves the analysis of an extract from the leaves of *Silphium albiflorum* Gray, a member of the Asteraceae plant family found only in central and north-central Texas.[11] This analysis represents the more typical case in which there is little prior knowledge as to the flavonoid contents of the sample. The extract used was known to contain monoglycosyl flavonoids, but ambiguous results were obtained by NMR analysis due to the difficulty in isolating each component. This application tested the ability of the new LC-MS/metal complexation method to provide conclusive compound identifications when conventional characterization methods failed.

5.2 EXPERIMENTAL

5.2.1 Materials

Manganese (II) chloride was purchased from Aldrich (Milwaukee, WI). Quercetin 3-O-galactoside (hyperoside) was purchased from Extrasynthèse (Genay, France). All materials were used without further purification.

5.2.2 Flavonoid Extraction from Apple Peel

Organic Fuji apples (*Malus domestica* Borkh. cv. Fuji) were purchased from a local supermarket and refrigerated until used. The extraction procedure was adapted from one described by Dick et al.[1] One apple was washed and peeled, and the peel (~15 g) was immersed in liquid nitrogen before being chopped in a blender. The finely chopped apple peel was added to a flask containing 100 mL methanol. The flask was heated to 50 °C and stirred for 30 minutes. The contents of the flask were then filtered through a fritted glass funnel, and the extract solvent was passed through 0.2 μm PTFE syringe filters (Fisher, Pittsburgh, PA). This clean-up procedure proved ineffective as a solid precipitate formed upon refrigeration of the sample. The methanol was then evaporated with nitrogen, and the extract was redissolved in 8 mL of water with 0.33% formic acid (mobile phase A). A C18 SepPak (Waters, Milford, MA) was used to purify and concentrate the flavonoid species in the extract. The cartridge was conditioned with 10 mL of acetonitrile with 0.33% formic acid (mobile phase

B) followed by 8 mL mobile phase A. The extract was loaded and washed with 5 mL of 98:2 A:B. The flavonoid species were eluted with 2 mL of 67:33 A:B. The sample was refrigerated until analyzed, and was injected onto the HPLC column without further treatment.

5.2.3 Flavonoid Extraction from Onions

Red onions (*Allium cepa* L., unknown cultivar) were purchased from a local supermarket and refrigerated until used. The extraction procedure was adapted from one described by Marotti and Piccaglia.[6] An onion was peeled and sliced, then immersed in liquid nitrogen before being chopped in a blender. Approximately 20 g of the chopped onion was added to a flask containing 50 mL of 50:42:8 methanol:water:acetic acid. The flask was covered and refrigerated for 48 hours. The extraction mixture was then filtered through a fritted glass funnel, and the solvent was evaporated with nitrogen. The extract was redissolved in 15 mL of mobile phase A. A solid phase extraction procedure similar to the one described for the apple peel extract was used to purify and concentrate the onion extract. The sample was refrigerated until analysis, and was diluted tenfold with methanol prior to injection onto the HPLC column.

5.2.4 Flavonoid Glycosides from *Silphium Albiflorum*

The extraction was performed by Jeffrey Williams and Małgorzata Wojcińska of the University of Texas Department of Biology and Poznań University of Medical Sciences, respectively. The procedure is detailed elsewhere.[12] One fraction containing several flavonoid glycosides proved difficult to purify enough for NMR analysis, so LC-MS^n with metal complexation was used as an alternate identification method. Approximately 0.1 mg of this fraction was dissolved in 0.5 mL methanol, and this solution was injected onto the HPLC column without further treatment.

5.2.5 LC-MS Conditions

Chromatography was performed using a Waters Alliance 2695 HPLC system (Milford, MA). The column was a Waters Symmetry C18, 2.1 x 50 mm, 3.5 μm particle size, with a guard column. The flow rate was 0.3 mL/min, and an injection volume of 10 μL was used for most experiments; however 40 μL injections were used to verify minor components of the *Silphium albiflorum* extract. For the apple peel extract, the two-component mobile phase system (see Section 5.2.2) was held isocratically at 12% B for 26 minutes, then was increased to 98% B over 2 minutes. For the onion extract, the gradient began at 10% B, increased to 25% B over 10 minutes, then to 95% B over 1.5 minutes. For the *Silphium albiflorum* extract, an isocratic separation method using 14% B for 16

minutes was employed. An ultraviolet (UV) detector monitored the column effluent at 280 nm. The column effluent was directed into the LCQ mass spectrometer without splitting. For negative ion mode experiments, the following MS conditions were used: spray voltage, -4.5 kV; sheath gas, 20 units; auxiliary gas, 5 units; heated capillary temperature, 200 °C; capillary voltage, -45 V; tube lens offset, -45 V. When flavonoid glycoside/manganese complexes were analyzed, 500 μM $MnCl_2$ dissolved in methanol was added at 20 μL/min via a mixing tee between the UV detector and the mass spectrometer. (A diagram of the instrumental set-up was shown in Figure 2.1.) In these cases the positive ion mode was employed, using the same spray conditions with the following exceptions: spray voltage, +4.5 kV; capillary voltage, +44 V; tube lens offset, +35 V.

5.3 Results and Discussion

5.3.1 Sensitivity Assessment

When a new analytical chemistry technique is developed, it is customary to measure the sensitivity of the new method. The limit of detection (LOD) is usually reported, and is defined as the amount or concentration of analyte required to produce a signal-to-noise ratio of 3. But in the case of these metal complexation methods, a true LOD is not the most applicable figure of merit. It is not sufficient to simply observe the metal complexes; the complexes must also be

fragmented by CID to yield a sufficiently recognizable pattern in order to make the proper structural determination. CID fragmentation processes always involve some signal loss due to scattering, so it is more important to evaluate the quality of the fragment ion spectrum than the signal-to-noise ratio of the parent complex.

In order to estimate the sample requirements, a standard of hyperoside (quercetin 3-O-galactoside) was chosen because this compound requires two fragmentation steps for identification of the saccharide moiety. A determination can be made as to whether the distinguishing fragment ions are clearly observed from injections of different amounts of sample (Figure 5.1). A 200 pmol injection of hyperoside yields a clean MS/MS spectrum with the –Hex fragment ion clearly visible. There is a small amount of noise in the MS^3 spectrum, but recognition of the three expected product ions, including the key –(Agl+102) fragment of m/z 416 that allows the identification of the galactose moiety, is unimpeded. In the 100 pmol injection, the MS/MS data is still fairly free of noise, while the m/z 416 ion is just visible above the noise in the MS^3 spectrum. In the 50 pmol injection, there is more noise in the MS/MS spectrum, though the –Hex fragment is still clear. However, the quality of the MS^3 spectrum is so low that it would be difficult to distinguish the relevant fragment ions from the noise. Hence, in this example 100 pmol (50 ng) is deemed to be the lowest analyzable quantity below which identification becomes uncertain. This experiment provides a ballpark assessment of the sensitivity of the LC-MS metal complexation methods, though

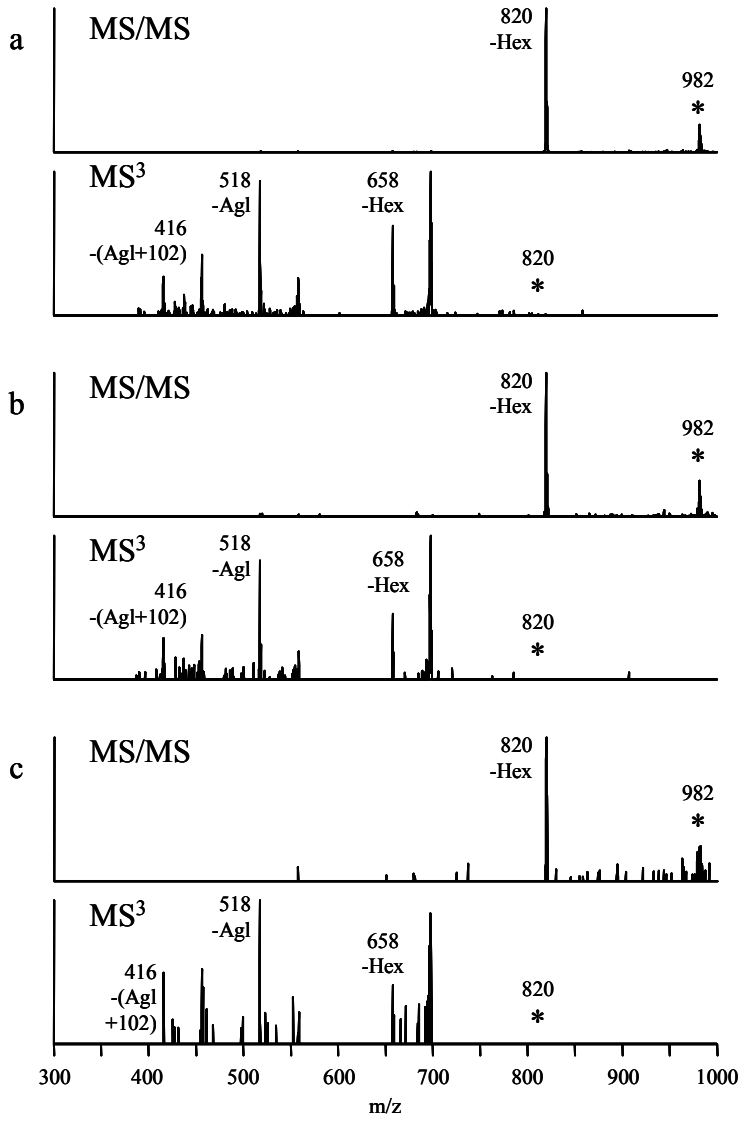


Figure 5.1: LC-MS^n spectra of hyperoside (quercetin 3-O-galactoside) complexes, [Mn(II) (FG-H) (FG)]$^+$ using varying injection amounts. a) 200 pmol injection; b) 100 pmol injection; c) 50 pmol injection. Some unlabeled peaks in the MS^3 spectra are post-CID acetonitrile adducts (+41 Da).

there will be variability be compound, by tuning, and by the number of fragmentation steps required to identify the flavonoid glycoside.

5.3.2 Apple Peel Extract

Apples were a special target of interest because they are known to contain several monoglycosyl flavonoid isomers that differ only by the identity of the saccharide moieties, and thus make an excellent test subject for the newly developed methods. The extracts were examined using both negative ion mode and positive ion mode (for the Mn complexes) because complementary information was obtained from each type of experiment. The extracts were prepared as described in Section 5.2.2.

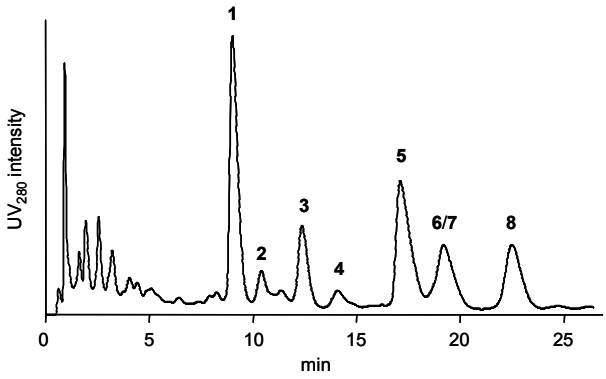


Figure 5.2. UV chromatogram of the Fuji apple peel extract, 280 nm. Flavonoid components are numbered.

The UV chromatogram of the Fuji apple peel extract (Figure 5.2) showed eight major flavonoid components, labeled **1** through **8**. LC-MS^n analysis in the negative ion mode provides the molecular weights of the flavonoid glycosides (full scan mode) and the aglycon portions (MS/MS mode), as well as the number and weight of the saccharide moieties for each compound (MS/MS mode). For

example, a loss of 162 Da indicates an O-hexoside, a loss of 146 Da indicates an O-deoxyhexoside, and a loss of 132 Da indicates an O-pentoside.[13] Losses associated with cross-ring saccharide cleavages, such as 120 Da or 90 Da, are indicative of C-glycosylation.[14] Upon sequential stages of fragmentation (MS^n), the saccharide moieties can be enumerated and some of their possible identities can be eliminated based on molecular weight. Following the loss of all of the saccharide moieties, the weight of the aglycon portion is obtained, and the aglycon may be identified by comparing the fragmentation pattern with standards or by elucidating its structure based on well-known dissociation pathways of the flavonoid aglycons.[13,15-18] The process of determining this information for the eight major flavonoid species observed in the Fuji apple peel extract is summarized in Scheme 5.1. Compounds **1** through **7** all lose a single saccharide moiety, leaving behind a deprotonated aglycon with mass 301 Da. Further

1: 463 $\xrightarrow{-162}$ 301 (aglycon)
2: 463 $\xrightarrow{-162}$ 301 (aglycon)
3: 433 $\xrightarrow{-132}$ 301 (aglycon)
4: 433 $\xrightarrow{-132}$ 301 (aglycon)
5: 433 $\xrightarrow{-132}$ 301 (aglycon)
6: 433 $\xrightarrow{-132}$ 301 (aglycon)
7: 447 $\xrightarrow{-146}$ 301 (aglycon)
8: 567 $\xrightarrow{-(132+162)}$ 273 (aglycon)

Scheme 5.1. Summary of negative ion mode MS/MS structural data from the flavonoid components in the Fuji apple peel extract.

fragmentation of m/z 301 identifies the aglycon as quercetin based on comparison to published spectra[15] and to the CID spectrum of a commercial standard. Thus **1** and **2** are quercetin O-hexosides, **3** through **6** are quercetin O-pentosides, and **7** is a quercetin O-deoxyhexoside. **8** was later determined to be a member of the chalcone family, a minor flavonoid class that is outside the scope of the current study.

Negative ion mode LC-MS^n provides useful structural information but does not reveal the nature of the saccharide moieties. At this point one would typically need to isolate each flavonoid glycoside and undertake NMR characterization to identify the saccharide moieties. Another approach would be to inject standards to check for matching retention times (although many flavonoid glycosides are not commercially available). In this study, LC-MS^n with post-column Mn(II) complexation was used to probe the identities and locations of the saccharides. As discussed in Section 3.3.4, complexes of the form $[Mn(II)(FG\text{-}H)(FG)]^+$ are effective in determining the glycosylation site of flavonoid glucosides, and of flavonoid galactosides conjugated at the 3 position (see Section 4.3.2). LC-MS/MS of these 2:1 Mn(II) complexes of **1** and **2** in each case yielded a single fragment stemming from the loss of one hexose residue. This is the hallmark of 3-O-glycosylation. A second stage of CID undertaken on this fragment ion resulted in spectra that differentiate the two analytes (Figure 5.3). The diagnostic loss of one aglycon unit plus 102 Da was observed only for **1**.

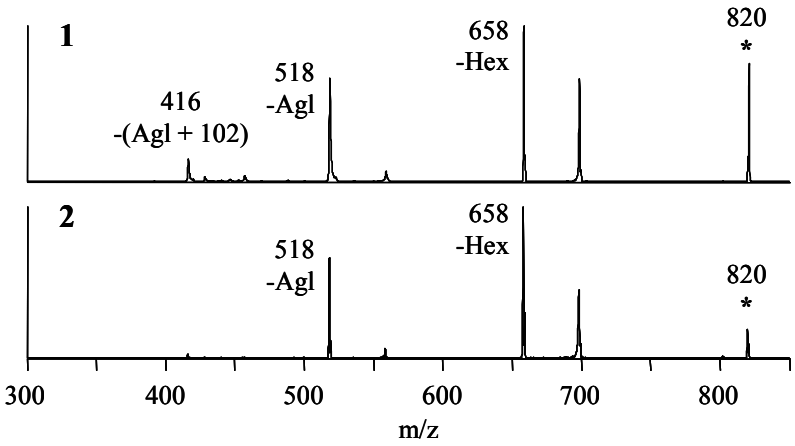


Figure 5.3. MS^3 of [Mn(II) (FG) (FG-H)]$^+$ complexes involving components **1** and **2** of the Fuji apple peel extract. The CID energy was 24%. Some of the unlabeled peaks in the spectra are post-CID adducts of acetonitrile (+41 Da).

Therefore **1** was determined to be a galactoside and **2** was identified as a glucoside. Upon integration of all of this data, **1** and **2** were identified as quercetin 3-O-galactoside and quercetin 3-O-glucoside. This is in agreement with the observation that flavonoid galactosides generally elute before flavonoid glucosides.[2,19,20]

Of the quercetin pentosides, only **3** and **5** gave Mn(II) complexes of sufficient abundance for analysis. In order to determine the identity of the saccharides, LC-MS/MS was performed on the 3:1 complex, [Mn(II) (FG-H) $(FG)_2]^+$ (Figure 5.4). The CID spectra of these complexes identify **3** as a xyloside and **5** as an arabinofuranoside. Fragmentation of **7** was not necessary to identify

this analyte as a quercetin O-rhamnoside because rhamnose is the only saccharide of molecular weight 146 known to form natural flavonoid conjugates.[13,21]

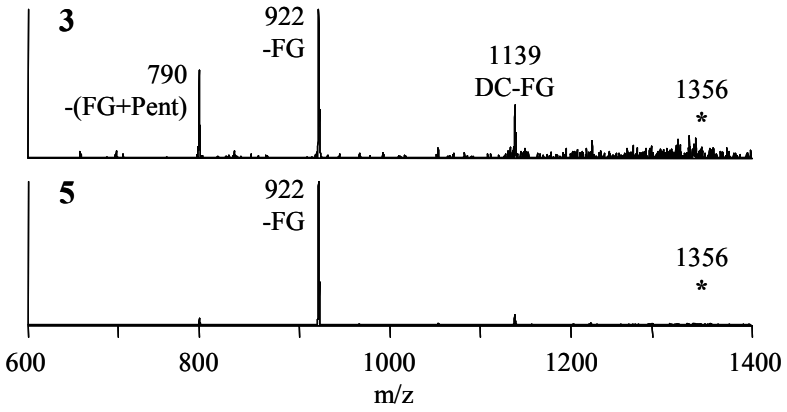


Figure 5.4. MS/MS spectra of [Mn(II) $(FG)_2$ (FG-H)]$^+$ complexes involving components **3** and **5** of the Fuji apple peel extract. The CID energy was 17%. The ions labeled as DC-FG refers to ([2 Mn(II) $(FG)_4$ $(FG-H)_2]^{2+}$ – FG).

Retention time matching with commercial standards confirmed the identities of **1** and **2**. The retention times of **3**, **5** and **7** matched those of quercetin 3-O-xyloside, quercetin 3-O-arabinofuranoside, and quercetin 3-O-rhamnoside, respectively. The retention order of these five compounds agrees with other published studies of apple flavonoids.[1-5] The Mn complexation method provided no additional information on compounds **4**, **6** and **8**. Using as a guide a particularly detailed study of apple waste components by Sánchez-Rabaneda et al.,[3] it is believed that **4** is quercetin 3-O-arabinopyranoside and **8** is phloretin 2′-O-xyloglucoside (mistakenly labeled phloretin-2-O-xyloglucoside in the

reference). From the same source, some of the early eluting peaks are thought to be various cinnamic acid derivatives. Like the present case, Sánchez-Rabaneda et al. observed an unidentified quercetin O-pentoside (**6**) co-eluting with quercetin 3-O-rhamnoside. It is speculated that **6** may be an apiose derivative of quercetin.[5]

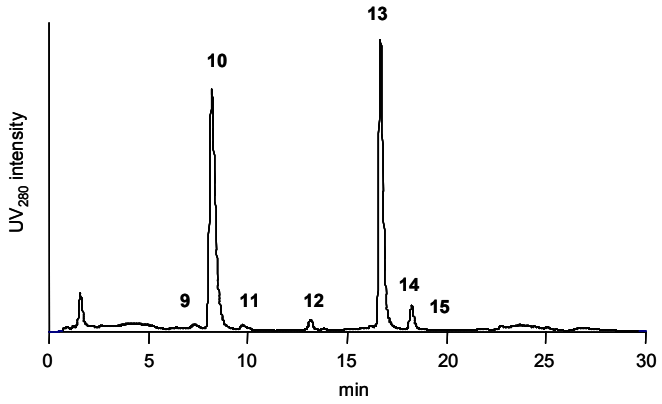
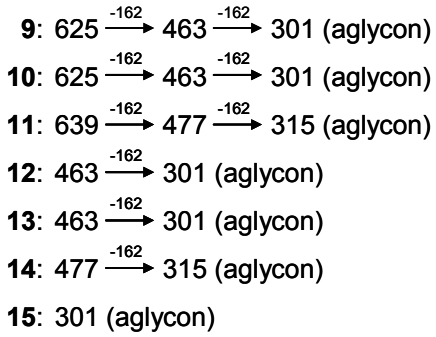


Figure 5.5. UV chromatogram of the red onion extract, 280 nm. Flavonoid components are numbered.

5.3.3 Onion Extract

There were at least seven different flavonoid species present in the onion extract, labeled **9** through **15** in order of elution (Figure 5.5). As with the apple extract, negative ion mode LC-MS^n was used to determine the weights of the aglycon portions and the number and types of glycosyl groups. This data is summarized in Scheme 5.2. The deprotonated aglycon of m/z 301 Da (**9**, **10**, **12**, **13** and **15**) was determined to be quercetin based on comparison of the fragmentation pattern to published spectra[15] and to the CAD spectrum acquired



Scheme 5.2. Summary of negative ion mode MS/MS structural data from the flavonoid components in the red onion extract.

from a commercial standard. **15** was further confirmed to be quercetin based on comparison of the retention time with a commercial standard. The deprotonated aglycon of m/z 315 (**11** and **14**) was similarly determined to be isorhamnetin based on comparison of the fragmentation pattern with published spectra[2] and with a commercial standard. **9**, **10** and **11** all contain two hexose moieties while **12**, **13** and **14** have one each. This agrees with the observation that additional saccharide moieties increase the polarity of a flavonoid glycoside, thus leading to faster elution in reversed-phase chromatography.[19]

To reveal the identities, locations, and configurations of the saccharide moieties of these analytes, post-column Mn complexation was performed. **9** and **11** did not form sufficient quantities of the complexes due to their low abundance and **15** (a flavonoid aglycon, quercetin) was fully characterized in the negative ion mode, so only **10**, **12**, **13** and **14** were studied using Mn complexation. The

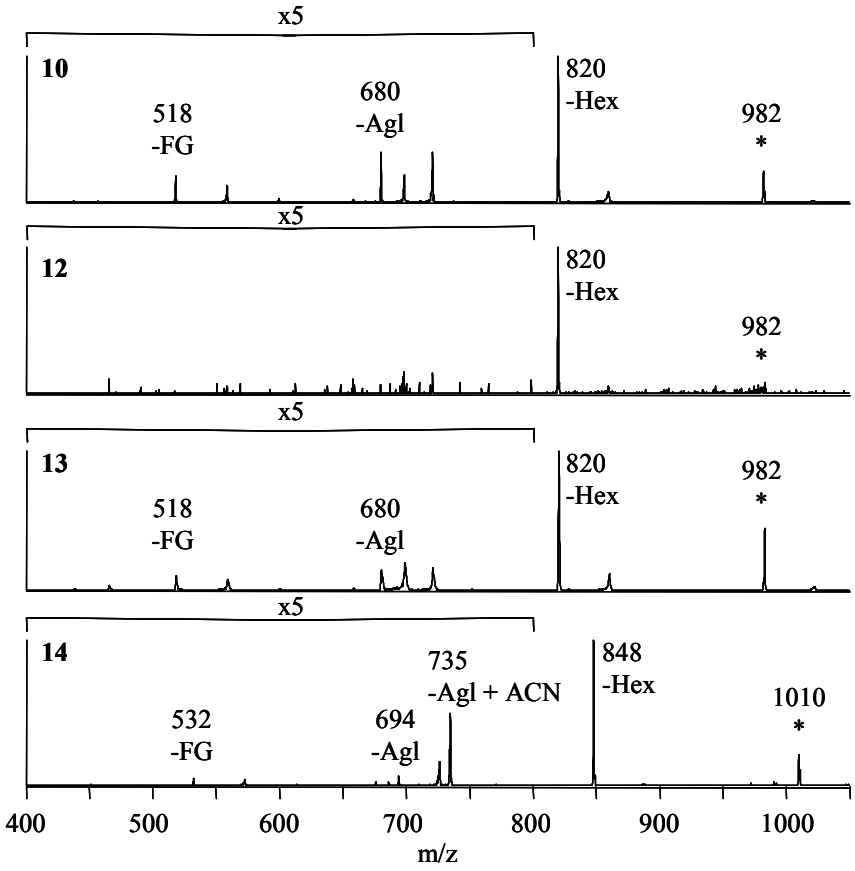


Figure 5.6. CID spectra of $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes involving components of the red onion extract. The CID energy was 21-22%. Some unlabeled peaks are post-CID adducts of acetonitrile (+41 Da) or water (+18 Da).

MS/MS spectra of the 2:1 complexes (Figure 5.6) were collected in order to determine the glycosylation sites of these compounds. The lack of any cross-ring saccharide cleavage fragments suggested that all four are 3-O- or 4′-O-glycosides. Fragments indicative of 4′-O-glycosides include the loss of one flavonoid glycoside molecule and the loss of an aglycon portion, whereas 3-O-glycoside complexes generally display only one major loss of a saccharide. **13** clearly

shows these additional fragments, while they do not appear for **12**; therefore **13** is suggested to be a 4′-O-glycoside while **12** is assigned as a 3-O-glycoside. **14** also shows these diagnostic fragment ions, so it is identified as a 4′-O-glycoside of isorhamnetin. **10** is a special case: diglycosyl flavonoids are generally outside the scope of this study but **10** nonetheless formed complexes of the type [Mn(II) (FG-H) (FG)]$^+$ of m/z 1306. Performing sequential stages of fragmentation on this complex yielded an ion m/z 982 after the loss of two hexose moieties, presumably one from each flavonoid glycoside molecule. At this point what remained was effectively a 2:1 monoglycosyl flavonoid complex, so the position of the remaining hexoses could be studied using the same techniques as discussed above. The fragments obtained from m/z 982 match the ones from **13**; thus it was concluded that at least one of the hexose moieties of **10** is at the 4′-O position.

The spectral quality is somewhat worse for these LC-MS experiments compared to the direct infusion experiments described in Chapter 3. Considering that the diagnostic ions in Figure 5.6 are of fairly low abundance, supporting information regarding the glycosylation site is desirable. Additional evidence is obtained through a judicious choice of CID energy. As described in Section 3.3.4, the amount of CID energy required to fragment the [Mn(II) (FG-H) (FG)]$^+$ complexes of flavonoid 4′-O-glucosides is higher than that of the analogous flavonoid 3-O-glucosides. More specifically, the CID energy required to reduce the parent ion of the 3-O-glycoside complexes to 5-10% overall abundance is

below 19%, while for the 4′-O-glycoside complexes it is above 22%. If a collision energy between these values is chosen, the survival rate of the parent ion can be used as a useful piece of confirming evidence. A CID energy of 21-22% was used to collect the data shown in Figure 5.6. It was observed that a significant amount of the parent ions survive for **10**, **13** and **14**, which is consistent with their assignments as 4′-O-glycosides. The parent ion of **12** does not survive, supporting its identification as a 3-O-glycoside. Additionally, the elution order of these compounds can be used as evidence.[19,22] Experience has shown that flavonoid 3-O-glycosides generally elute earlier than flavonoid 4′-O-glycosides (to be discussed further in Section 7.3.3). This is consistent with the assignment of **12** and **13** as 3-O- and 4′-O-glycosides of quercetin, respectively.

Finally, a second stage of fragmentation determined the identity of the hexose moiety of **12**. When the ion of m/z 820 was activated, the fragment ion corresponding to the loss of quercetin aglycon plus 102 Da was not observed (Figure 5.7). It is clear that the hexose cannot be galactose, and is therefore assigned as glucose. **10**, **13** and **14** also lack the diagnostic ion for galactose, but this evidence is inconclusive because no standards are available to determine whether the Mn complexes of 4′-O-galactosides can be differentiated from 4′-O-glucosides in the same manner as at the 3-O-position.

In summary, the peak assignments based solely on these LC-MS methods are: **9** – quercetin dihexoside, **10** – quercetin dihexoside (with at least one of the

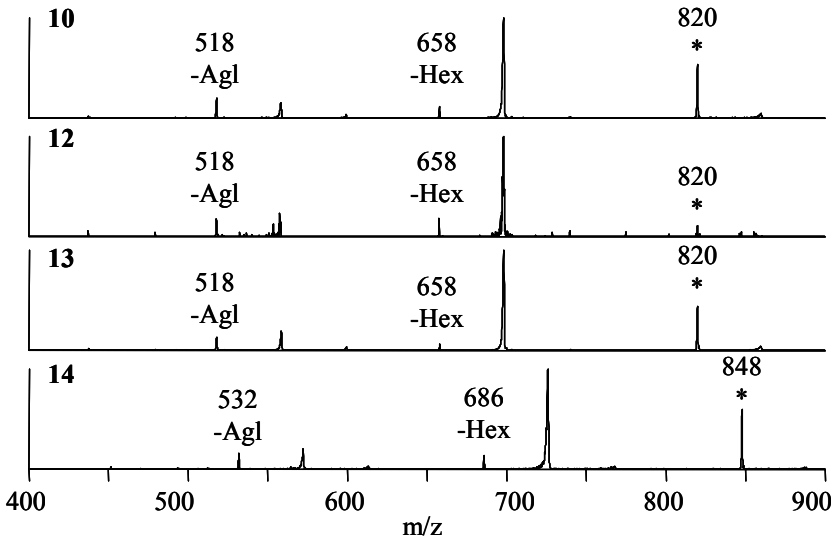


Figure 5.7. MS^3 spectra of $[Mn(II)\ (FG)\ (FG\text{-}H)]^+$ complexes involving components of the red onion extract. The CID energy was 23% in all cases. Some unlabeld peaks are post-CID acetonitrile adducts (+41 Da).

hexoses located at the 4′ position), **11** – isorhamnetin dihexoside, **12** – quercetin 3-O-glucoside, **13** – quercetin 4′-O-hexoside (possibly glucoside), **14** – isorhamnetin 4′-O-hexoside (possibly glucoside), **15** – quercetin. Standards of quercetin 3-O-glucoside and quercetin had the same retention times as **12** and **15**, respectively, confirming those identifications. A quercetin 4′-O-glucoside standard had the same retention time as **13**. No standard for isorhamnetin 4′-O-glucoside was available, but isorhamnetin 3-O-glucoside did not match the retention time of **14**. The accuracy of these assignments was assessed by comparison with several previously published studies of onion flavonoid

components.[6,8-10] Based on the literature, it is believed that **12**, **13**, **14** and **15** have been identified correctly using the LC-MS method with Mn complexation. **10** is almost certainly quercetin 3,4′-di-O-glucoside,[10] and **9** may be quercetin 7,4′-di-O-glucoside.[9] **11** may be isorhamnetin 3,4′-di-O-glucoside, which has been reported as a minor flavonoid glycoside in onions.[7]

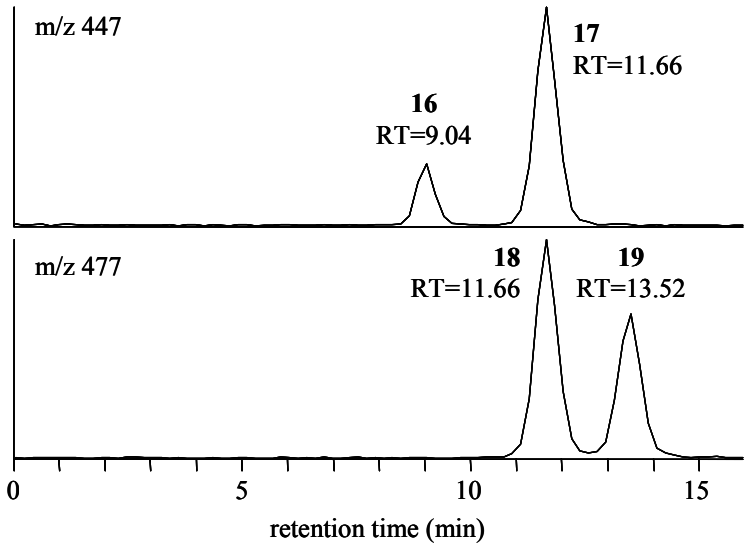


Figure 5.8. Mass chromatograms of flavonoid components of a *Silphium albiflorum* fraction, acquired in negative ion mode with selected ion monitoring.

5.3.4 *Silphium albiflorum* Extract

LC-MS analysis of the *Silphium albiflorum* extract revealed four components, two (**16** and **17**) of molecular weight 448 and two (**18** and **19)** of molecular weight 478 (Figure 5.8). In negative ion mode, CID resulted in the loss of one hexose moiety (-162 Da) from each compound. **16** and **17** were

determined to be kaempferol derivatives and **18** and **19** were identified as isorhamnetin derivatives by comparing the fragmentation patterns of the aglycon portions with commercial standards. In order to determine the locations and identities of the hexose moieties, LC-MS^n with post-column manganese complexation was used. Performing CID on the $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes yielded a single fragment ion corresponding to the loss of one hexose moiety (Figure 5.9 a). This result is indicative of 3-O-glycosylation. Further dissociation of these initial fragment ions allowed the hexose moieties to be identified (Figure 5.9 b). Losses of the second hexose moiety and of an aglycon

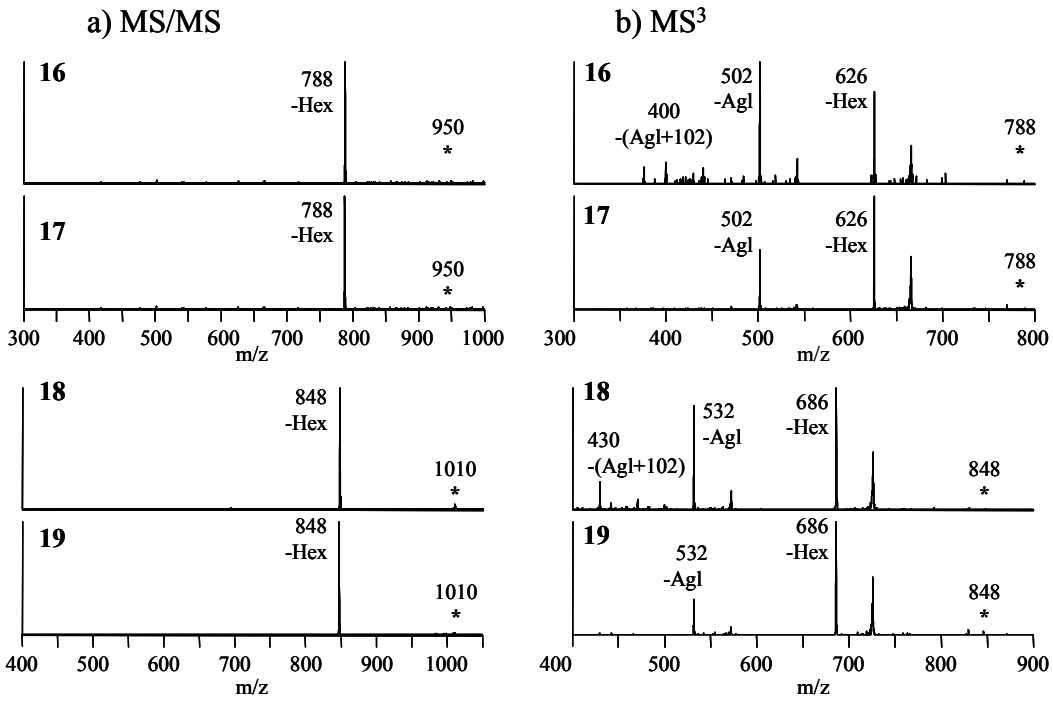


Figure 5.9. (a) MS/MS and (b) MS^3 spectra of the $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ complexes involving components of the *Silphium albiflorum* extract. Some unlabeled peaks are post-CID acetonitrile adducts (+41 Da).

portion were observed for all four complexes. However an additional fragment ion corresponding to the loss of an aglycon portion plus 102 Da was observed only for **16** and **18**. Thus the compounds in the extract were identified as: **16** – kaempferol 3-O-galactoside, **17** – kaempferol 3-O-glucoside, **18** – isorhamnetin 3-O-galactoside, and **19** – isorhamnetin 3-O-glucoside. The identities of **17** and **19** were confirmed by retention time comparison with commercial standards. No standards were available for **16** and **18**, but their identifications are supported by the observation that flavonoid galactosides generally elute slightly earlier than flavonoid glucosides using reversed-phase HPLC.[2,19,20]

5.4 CONCLUSIONS

Performing CID on complexes of the form $[Mn(II)\ (FG\text{-}H)\ (FG)]^+$ and $[Mn(II)\ (FG\text{-}H)\ (FG)_2]^+$ provides information on the glycosylation site and saccharide identity of these compounds based on unique and consistent fragmentation patterns. This method of identifying flavonoid glycosides was applied to the on-line LC-MS analysis of a Fuji apple peel extract, a red onion extract and an extract from *Silphium albiflorum*. Combined with information obtained from the deprotonated analytes, this method allowed the identification of several of the flavonoid glycosides in the extracts. Supporting evidence was obtained by using retention time comparison with commercial standards and knowledge of the elution order associated with various structural features. The

identifications were in agreement with the literature. Although complete identifications could not be made in all cases, significantly more structural information was obtained than was previously possible using conventional LC-MS techniques. Furthermore, metal complexation has been shown to be a promising approach to mass spectrometric differentiation of flavonoid glycosides even when standards are not available. It is hoped that further correlations between structure and dissociation behavior will be found such that full characterization of these and similar compounds will be possible using simple LC-MS techniques. This would be a significant advantage over the isolation and purification of each compound followed by NMR analysis that is currently the standard method for characterizing the saccharide moieties of flavonoid glycosides.

5.5 REFERENCES

(1) Dick AJ, Redden PR, Demarco AC, Lidster PD, Grindley TB. *Journal of Agricultural and Food Chemistry* **1987**, *35*, 529-531.
(2) Schieber A, Keller P, Streker P, Klaiber I, Carle R. *Phytochemical Analysis* **2002**, *13*, 87-94.
(3) Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Viladomat F, Bastida J, Codina C. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 553-563.
(4) Oleszek W, Lee CY, Jaworski AW, Price KR. *Journal of Agricultural and Food Chemistry* **1988**, *36*, 430-432.
(5) Lommen A, Godejohann M, Venema DP, Hollman PCH, Spraul M. *Analytical Chemistry* **2000**, *72*, 1793-1797.
(6) Marotti M, Piccaglia R. *Journal of Food Science* **2002**, *67*, 1229-1232.

(7) Tsushida T, Suzuki M. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 100-108.
(8) Park YK, Lee CY. *Journal of Agricultural and Food Chemistry* **1996**, *44*, 34-36.
(9) Ferreres F, Gil MI, Tomas-Barberan FA. *Food Research International* **1996**, *29*, 389-395.
(10) Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G. *Free Radical Research* **2001**, *35*, 941-952.
(11) Clevinger JA, Panero JL. *American Journal of Botany* **2000**, *87*, 565-572.
(12) Wojcinska M, Williams J, Mabry T, Ahmed AA, Davis BD, Toth G, El-Sayed NH, Matlawska I, Clevinger J. *Natural Product Communications* **2006**, *1*, 941-948.
(13) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
(14) Waridel P, Wolfender JL, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Journal of Chromatography A* **2001**, *926*, 29-41.
(15) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
(16) Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(17) Hughes RJ, Croley TR, Metcalfe CD, March RE. *International Journal of Mass Spectrometry* **2001**, *210/211*, 371-385.
(18) Kuhn F, Oehme M, Romero F, Abou-Mansour E, Tabacchi R. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.
(19) Robards K, Antolovich M. *Analyst* **1997**, *122*, 11R-34R.
(20) Schieber A, Keller P, Carle R. *Journal of Chromatography A* **2001**, *910*, 265-273.
(21) Cuyckens F, Shahat AA, Pieters L, Claeys M. *Journal of Mass Spectrometry* **2002**, *37*, 1272-1279.
(22) Huck CW, Buchmeiser MR, Bonn GK. *Journal of Chromatography A* **2002**, *943*, 33-38.

Chapter 6: Identification of Monoglucuronyl Flavonoid Metabolites in Urine, Plasma and Cell Cultures Using Metal Complexation and LC-MS^n

6.1 INTRODUCTION

Chapters 3, 4 and 5 covered structural identification of flavonoid glycosides, with applications in botanical and food analysis. This chapter and the next focus on the metabolic and biotransformational products of flavonoids. The investigation of metabolic pathways is a vital step in understanding the mechanism of action of bioactive molecules. Recent work has begun to shed light on the biotransformation of dietary flavonoids.[1] It is now known that while flavonols, flavanones and flavones are consumed in the diet mainly as glycoside conjugates, the saccharide portions are removed during absorption.[2-4] Because metabolism occurs rapidly, it is mainly the glucuronidated, sulfated and methylated derivatives that are present in plasma following consumption of flavonoids.[5,6] As a result of these findings, concerns have been raised about the reliability of much of the *in vitro* data on flavonoid bioactivity.[7,8] Most *in vitro* studies have involved either flavonoid aglycons or glycosides that are not present for any appreciable amount of time in the body, and the doses applied are often at much higher levels than are achieved *in vivo* after eating foods rich in flavonoids. It has therefore been suggested that *in vitro* studies of flavonoids should use metabolites rather than commercially available aglycons and the glycoside forms

found in foodstuffs.[7,8] However, the problem remains that far less is known about the *in vivo* metabolites than about their precursors, and relatively few analytical methods have been developed for studying these metabolites. The commercial availability of flavonoid metabolite standards is extremely limited.

As with all flavonoid derivatives, determining the substitution patterns of flavonoid glucuronides is a considerable challenge. Nuclear magnetic resonance (NMR) spectroscopy may be used to obtain this information,[9,10] but this technique is less applicable to dietary metabolites which are present in very low concentrations. A UV-Vis spectroscopic method[11] has also been used to determine the position of the glucuronide moieties on flavonoid metabolites,[12] but this is a complicated method involving the use of several shift reagents. Liquid chromatography/tandem mass spectrometry (LC-MS^n) is in many ways an ideal method for analyzing and identifying flavonoid metabolites due to its high sensitivity, applicability to complex mixtures, and ability to provide structural information. However, full structural characterization is often not possible by mass spectrometry (MS). While flavonoid glucuronides can be identified by the characteristic loss of the glucuronic acid moiety (-176 Da) upon dissociation, it has not yet been possible to determine the location of the glucuronic acid moiety by mass spectrometry.[13-17] Previous work has shown that the positions of saccharide moieties have a large effect on the bioactivity of flavonoids[18,19] and a similar effect was noted for conjugated metabolites.[12] Thus the ability to

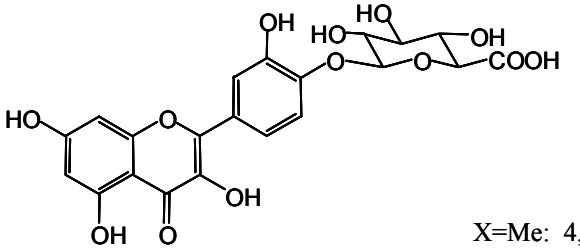
elucidate the structure of flavonoid metabolites as comprehensively as possible is extremely useful.

Metal complexation combined with tandem mass spectrometry was shown in earlier chapters to be an effective strategy for the structural identification of flavonoid glycosides. This chapter introduces metal complexation methods that allow the position of glucuronidation to be determined for flavonoid metabolites containing a single glucuronic acid moiety. Four isomeric quercetin monoglucuronides were differentiated based on characteristic fragmentation patterns observed upon collision-induced dissociation (CID) of the complexes. Moreover, consistent product ion signatures were found for complexes involving 7-O-glucuronides and 3-O-glucuronides in the flavonol, flavone, and flavanone groups. The metal complexation methods were adapted for the analysis of flavonoid glucuronides in complex biological matrices via LC-MS with post-column complexation. The four quercetin glucuronides were spiked into rat plasma in order to estimate the sensitivity of the method. Finally, several applications are presented. The *in vivo* human urinary metabolites of citrus flavonoids were identified following consumption of grapefruit juice and oranges, and the glucuronidated products of cell culture incubations were also identified.

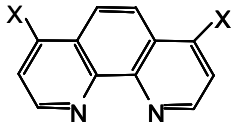
6.2 EXPERIMENTAL

6.2.1 Materials

Quercetin 3-O-glucuronide,[20] quercetin 3′-O-glucuronide,[20] quercetin 4′-O-glucuronide,[21] quercetin 7-O-glucuronide[21] and naringenin 7-O-glucuronide[22] were synthesized by Paul Needs and Paul Kroon at the Institute of Food Research (Norwich, UK). The above compounds were analyzed in their sodium salt form. Baicalin (baicalein 7-O-glucuronide) was purchased from Extrasynthèse (Genay, France). Kaempferol 3-O-glucuronide was purchased from Apin Chemicals (Abingdon, UK). Cobalt(II) bromide, 4,7-dimethyl-1,10-phenanthroline (4,7-dmphen), and 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen) were purchased from Aldrich (Milwaukee, WI). Structures of the phenanthroline ligands and a representative flavonoid glucuronide are depicted in Figure 6.1. Rat plasma was purchased from Pel-Freez Biologicals (Rogers, AR).



quercetin 4′-O-glucuronide
$C_{21}H_{18}O_{13}$, MW=478



X=Me: 4,7-dimethyl-1,10-phenanthroline
(4,7-dmphen) $C_{14}H_{12}N_2$, MW=208

X=Ph: 4,7-diphenyl-1,10-phenanthroline
(4,7-dpphen) $C_{24}H_{16}N_2$, MW=332

Figure 6.1. Chemical structures of selected compounds.

The urine samples from the grapefruit study were obtained from an earlier set of experiments, the details of which are published elsewhere.[17] The urine and plasma samples from the orange feeding study were obtained from the Institute of Food Research. Volunteers were given either 150 g of fresh orange segments or 300 g of orange juice as part of a standard breakfast. Blood and urine samples were collected from the volunteers. Full details of the study design will be available in an upcoming publication. The qualitative analysis of some of these samples is presented here. The CaCo-2 cell culture samples were prepared by Robert Barrington of the Institute of Food Research. All urine, plasma and cell culture samples were kept frozen until analysis.

6.2.2 Direct Infusion Experiments

The complexes were formed in methanol by adding $CoBr_2$, a flavonoid glucuronide, and either 4,7-dpphen or 4,7-dmphen, all at 10 μM concentrations. Experiments were performed on an LCQ Duo quadrupole ion trap mass spectrometer (Thermo Electron, Waltham, MA) with an ESI source. Solutions were introduced at a flow rate of 5 μL/min. The metal complexes were analyzed in the positive ion mode with a spray voltage of +5 kV and a heated capillary temperature of 150 °C. The gas flow rates, capillary voltage and tube lens offset were optimized for maximum signal intensity on a daily basis. An ion injection time of 10 msec was used for full scan mass spectra and 50 msec was used for

CID experiments. 100 individual scans were averaged for each spectrum. The CID collision energies were converted from the Normalized Collision Energy[23] values given by the LCQ mass spectrometer into absolute voltages applied to the ion trap during dissociation. Because the non-covalent complexes are relatively "fragile", isolation windows of 3-5 Da were required to obtain stable fragmentation spectra.[24] Such wide isolation windows have been reported for a variety of other non-covalent complexes examined by quadrupole ion trap mass spectrometry.[25-27] Product ion abundances are reported relative to the most abundant ion in the spectrum, which is designated as 100%.

6.2.3 Analysis of Spiked Rat Plasma

The four quercetin glucuronides were spiked into 500 μL of rat plasma to achieve 1 μM concentrations. The analytes were stabilized as described by Day et al.[28] by the addition of 50 μL of 0.65 mM acetic acid and 10 μL of 50 mM ascorbic acid as an antioxidant. After gentle mixing, 1 mL of acetonitrile was added to precipitate the plasma proteins. The sample was vortexed for 1 min and centrifuged for 10 min at 16000 g. The supernatant was collected and evaporated with nitrogen. The sample was reconstituted with 200 μL of water, 20 μL of 0.65 mM acetic acid and 20 μL of 50 mM ascorbic acid. Chromatography was performed on a Waters Alliance 2695 HPLC system (Milford, MA). The stationary phase was a Waters Symmetry C18 column, 2.1 x 50 mm, 3.5 μm

particles, with a matching guard column. An injection volume of 10 μL was used. A gradient method was employed using water with 0.05% formic acid as mobile phase A and methanol with 0.05% formic acid as mobile phase B. The gradient started at 35% B and increased to 75% B over 20 min, then increased to 95% B over 1 min, followed by re-equilibration. The flow rate was 0.1 mL/min. The UV signal was monitored at 370 nm. Post-column addition was performed by mixing the column effluent with a 5 μM methanolic solution of $CoBr_2$ and 4,7-dpphen flowing at 20 μL/min, which was added via a tee connection and controlled by a syringe pump. This mixture was introduced directly into the mass spectrometer, which was operated with a spray voltage of +4.5 kV and a capillary temperature of 200 °C. The gas flow rates, capillary voltage and tube lens offset were optimized for the highest signal intensity of the complex of interest. The automatic gain control (AGC) settings were used with a target of 2×10^7 ions and 5 microscan averaging.

6.2.4 Urine Analysis from Grapefruit Juice Study

Samples of human urine collected after the consumption of grapefruit juice were obtained in a previous study.[17] Only samples from one human volunteer were re-analyzed. The 4.25 hour timepoint was used because this was near the time of highest metabolite concentration excreted.[17] The frozen sample was thawed, and 400 μL of urine was added to 800 μL methanol and centrifuged

for 5 min at 16000 g. The supernatant was drawn off and evaporated with nitrogen. The sample was reconstituted in 1 mL of water with 0.05% formic acid (v/v). Solid phase extraction was performed using a C18 SepPak (Waters, Milford, MA). The SepPak was conditioned with methanol and 0.33% formic acid followed by water and 0.33% formic acid. The urine extract was loaded, washed with 90:10:0.33 water/methanol/formic acid, and eluted with 2 mL 25:75:0.33 water/methanol/formic acid. The chromatographic hardware, stationary phase and mobile phases were the same as described in Section 6.2.3. An injection volume of 10 µL was used. The isocratic separation method used 70% mobile phase B with a flow rate of 0.1 mL/min. The column effluent was sent to a UV detector operated at 280 nm prior to MS analysis. Post-column addition and analysis of the metal complexation was performed as described in Section 6.2.3.

6.2.5 Urine Analysis from Orange Intervention Study

2.5 mL aliquots of urine were added to 7.5 mL of methanol to effect protein precipitation. The mixture was vortexed and centrifuged for 15 min at 1380 g. The supernatant was collected and evaporated with nitrogen down to approximately 500 µL. The chromatographic hardware, stationary phase and mobile phases were the same as described in Section 6.2.3. Injection volumes of 20-40 µL were used. The gradient began at 25% B, increased to 30% B over 10

min, then increased to 50% B over 10 min, then to 100% B over 5 min, and was held constant at 100% B for 2 additional min. The flow rate was 0.3 mL/min throughout. For negative ion mode experiments, a spray voltage of -4.5 kV was used. The conditions used for metal complexation experiments were as described in Section 6.2.3.

6.2.6 Analysis of Cell Culture Extracts

CaCo-2 cell cultures were treated with kaempferol or galangin to produce glucuronidated and sulfated metabolites. The synthesis and extraction of these metabolites was performed at the Institute of Food Research, and details of these procedures will be available in a forthcoming publication. 10 μL aliquots of the extracts were injected and analyzed by LC-MS. All chromatography and mass spectrometry parameters were the same as described in Section 6.2.5.

6.3 Results and Discussion

6.3.1 Direct Infusion Experiments

Before focusing on metal complexation, the ability to differentiate isomeric flavonoid glucuronides based on tandem MS of the protonated or deprotonated species was evaluated. Because most of the standards were analyzed in their salt forms, these compounds provided very good signal intensity in the negative ESI mode. The MS/MS spectra of the $[FG-H]^-$ ions, however,

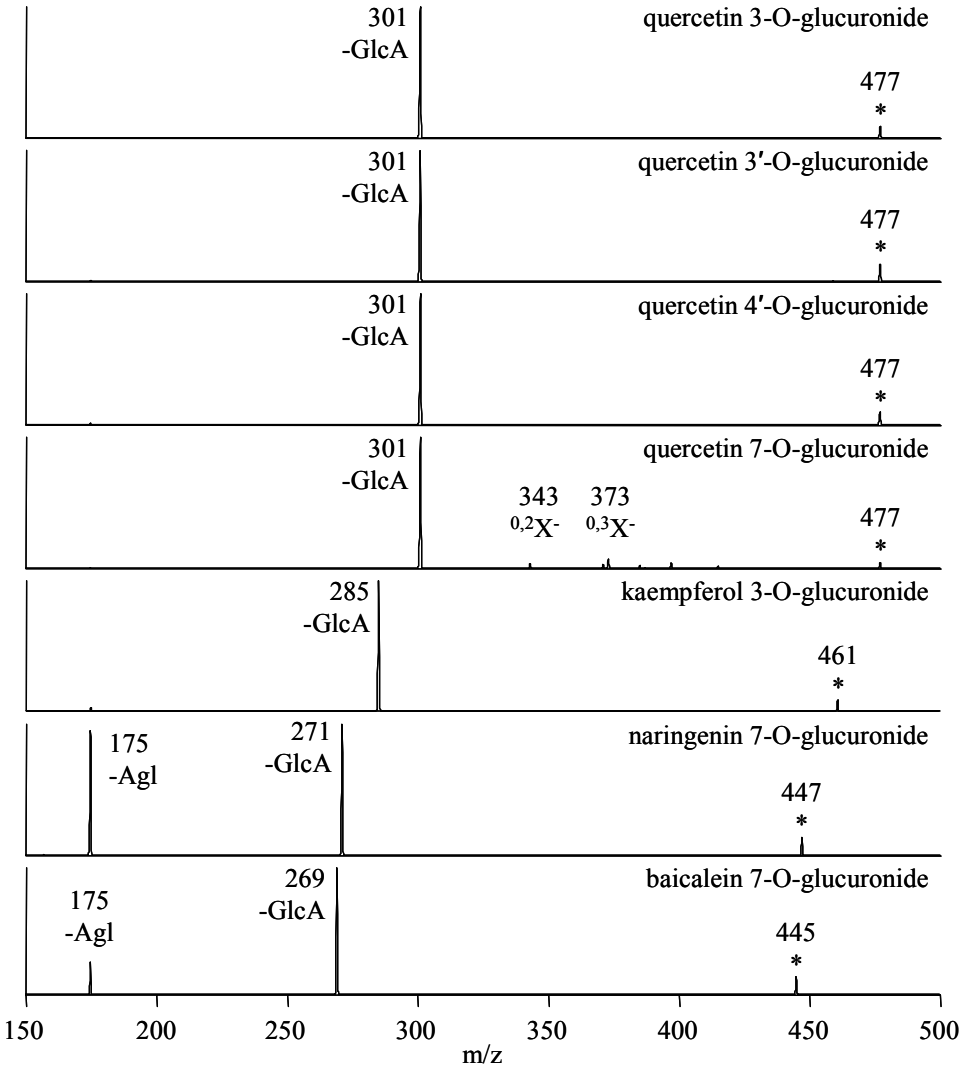


Figure 6.2. MS/MS spectra of the $[FG - H]^-$ species. The CID energy is 20-23% (0.48-0.56 V).

showed few distinguishing features (Figure 6.2). Three of the quercetin glucuronides yielded only one significant product ion (>5% relative abundance) resulting from the loss of the glucuronic acid moiety. It is impossible to determine the position of glucuronidation based on this evidence. Further stages

of dissociation (MS^n) were not helpful. Quercetin 7-O-glucuronide provided additional product ions due to cross-ring cleavages[29] of the glucuronic acid moiety, such as $^{0,3}X^-$ and $^{0,2}X^-$, but these extra product ions were not observed in the CID spectra of the other flavonoid 7-O-glucuronides. Furthermore, naringenin 7-O-glucuronide and baicalein 7-O-glucuronide both displayed a prominent loss of the aglycon portion of the molecule, which did not occur for quercetin 7-O-glucuronide. Ideally, a distinctive fragmentation pattern would be indicative of a particular site of glucuronidation regardless of the aglycon portion (which can be identified by other MS strategies). CID of the deprotonated flavonoid glucuronides does not provide such consistent indicators. The protonated species were also examined, but they proved difficult to observe as two protons must be transferred to the negatively-charged analytes to produce singly-charged positive ions. The positively-charged analytes could not be observed consistently even with the addition of acid, so attempts to work with these species were abandoned.

Given the similarity between flavonoid glucosides and flavonoid glucuronides, it was hypothesized that a metal complexation approach[30-36] might be effective in determining the position of the glucuronide moiety, but the intrinsic negative charge on the flavonoid glucuronides proved to be a major obstacle in emulating these earlier methods. Divalent metal cations like Mn(II) and Mg(II), which had been so useful in previous applications,[35,36] did not form

sufficiently abundant complexes with the analytes. This may be explained by the formation of neutral complexes of the type $[M(II)\ (FG\text{-}H)_2]^0$, which are "invisible" to the mass spectrometer. The metal ions Fe(III) and Al(III) were expected to form $[M(III)\ (FG\text{-}H)_2]^+$ products, but these metals also failed to provide observable complexes. Monovalent metals such as Li and Na would form neutral adducts $[M(I)\ (FG\text{-}H)]^0$ and so were not suitable candidates in the positive ESI mode, but the $[M(I)\ (FG\text{-}H)_2]^-$ species were quite abundant in the negative ESI mode. However, identical fragmentation profiles were obtained upon CID of isomeric species.

The formation of neutral flavonoid glycoside/metal complexes is a dead end for MS strategies, and one solution has been found based on the use of auxiliary ligands.[30] A neutral ligand replaces a negatively-charged flavonoid conjugate in the complex, ensuring an overall net positive charge. The use of auxiliary ligands in flavonoid glycoside/metal complexes has been shown to improve the sensitivity of analysis,[30] and the ligand itself plays a vital role in determining how the complex will dissociate.[32] Phenanthroline-based ligands had been fruitful in the past, so they were selected for the present application. Two different auxiliary ligands, 4,7-dmphen and 4,7-dpphen, were found that formed complexes with the flavonoid glucuronides and cobalt(II) while also allowing isomer differentiation by CID. Nickel(II) formed similar complexes, but the fragmentation results were rather less useful than with cobalt(II).

The best metal complexation mode found for differentiating the four quercetin monoglucuronide isomers involves the use of complexes of the form [Co(II) (FG-H) (4,7-dmphen)]$^+$. Dissociation of these complexes by CID results in four different fragmentation patterns, each with a unique set of product ions (Figure 6.3). The differentiation is based on the presence or absence of three key product ions: m/z 442 (loss of quercetin aglycon), m/z 536 (loss of the 4,7-dmphen molecule), and m/z 568 (loss of the glucuronic acid moiety). The quercetin-3-O-glucuronide complex dissociates to yield only one significant ion (>5% relative intensity) of m/z 536, in contrast to the other quercetin glucuronide complexes, for which the ion of m/z 568 is the most abundant product ion. The

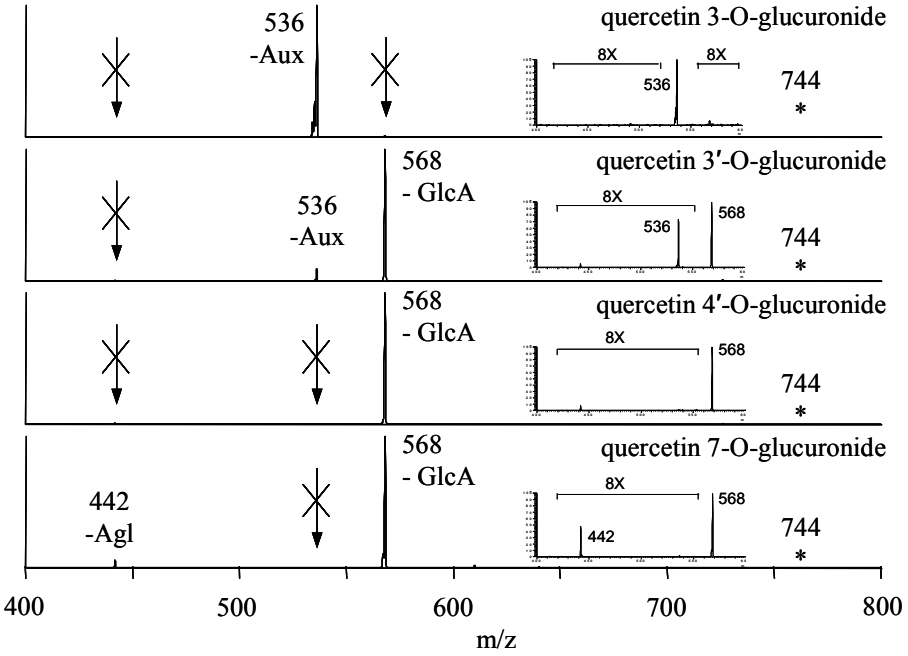


Figure 6.3. MS/MS spectra obtained from [Co(II) (FG-H) (4,7-dmphen)]$^+$. Insets show magnifications of the region between m/z 400 and 600. The CID energy is 40% (1.31 V).

loss of the glucuronic acid moiety is the only significant product ion yielded by the quercetin 4′-O-glucuronide complex, while the quercetin 3′-O-glucuronide complex and the quercetin 7-O-glucuronide complex yield small but significant losses of 4,7-dmphen and quercetin aglycon, respectively. It is hypothesized that the characteristic fragmentation patterns of these isomeric complexes are due to conformational differences which favor or disfavor the various dissociation pathways. This hypothesis is supported by the recent work of Clowers and Hill,[37] who used dual gate-ion mobility-quadrupole ion trap mass spectrometry to show that isomeric flavonoid glycosides complexed to various metal ions have different collisional cross-sections, an indication of conformational differences. Some complexes in that study assumed more than one stable conformation.

Energy variable CID experiments were undertaken to determine the dependence of these characteristic fragmentation pathways on collision energy (Figure 6.4). It was found that the key product ions appear for all complexes beginning at around 1.0 V applied to the trap during dissociation, and these ions are fairly stable in their appearance until at least 1.6 V. The insensitivity of the fragmentation pathways to collision energy is beneficial in that there is no need for fine control of this parameter in order to make the differentiation. In addition, these results are promising in terms of the ability to transfer this method to other tandem mass spectrometers which may be calibrated differently with respect to CID energies and performance.

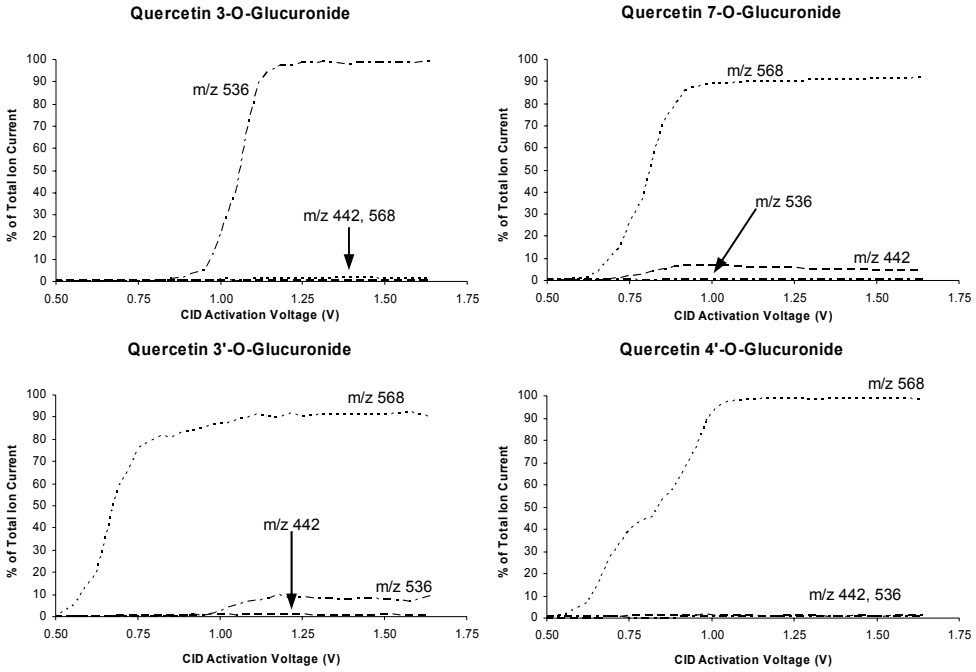


Figure 6.4. Energy variable CID results from [Co(II) (FG-H) (4,7-dmphen)]$^+$ complexes involving quercetin glucuronides. The precursor ion and minor product ions are omitted. Fragments are identified as: m/z 568 (loss of the glucuronic acid moiety), m/z 536 (loss of 4,7-dmphen), m/z 442 (loss of the aglycon portion).

While the [Co(II) (FG-H) (4,7-dmphen)]$^+$ complexes are extremely useful for differentiating the position of the glucuronide moiety of quercetin monoglucuronides, the same patterns of product ions were not observed for compounds derived from other flavonoids. Instead two other types of complexes were found that provide consistent fragments based on the glucuronidation position, regardless of the type of flavonoid. For example, complexes of the form [Co(II) (FG – H) (4,7-dmphen)$_2$]$^+$ yield consistent product ion profiles that reflect

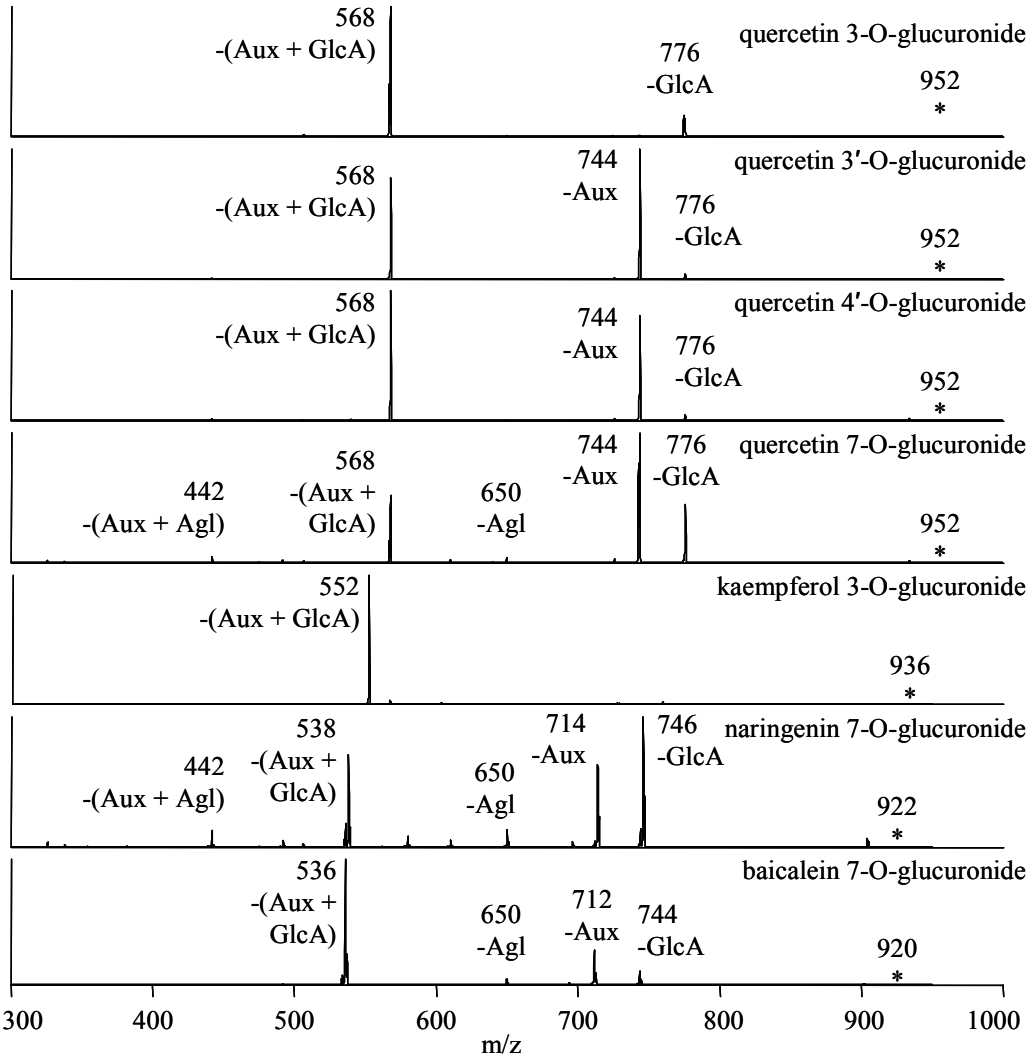


Figure 6.5. MS/MS spectra obtained from [Co(II) (FG-H) (4,7-dmphen)$_2$]$^+$. The CID energy is 40% (1.52-1.56 V).

the position of glucuronidation for flavonols, flavones and flavanones (Figure 6.5). The quercetin-3-O-glucuronide complex is differentiated from its isomers by the lack of a significant product ion corresponding to the loss of 4,7-dmphen (m/z 744). This ion is also absent from the CID spectrum of the kaempferol 3-O-

glucuronide complex, suggesting a consistent indicator of glucuronidation at the 3 position. The quercetin 7-O-glucuronide complex is differentiated from its isomers as it is the only one that yields abundant product ions stemming from the individual losses of 4,7-dmphen and of the glucuronide moiety. Only one of these two fragments appears with significant abundance in the MS/MS spectra of the other quercetin glucuronide complexes. Both product ions are also seen for the naringenin 7-O-glucuronide and the baicalein 7-O-glucuronide complexes. The presence of significant amounts of both product ions (loss of 4,7-dmphen and loss of the glucuronide moiety) in the MS/MS spectra of only the complexes involving the flavonoid 7-O-glucuronides suggests a potential general method for identifying flavonols, flavones and flavanones with a glucuronide moiety at position 7. The complexes involving quercetin 3′-O-glucuronide and quercetin 4′-O-glucuronide may be differentiated by the relative intensities of the ions of m/z 568 and m/z 744, but this type of differentiation is not expected to be applicable to flavonoid glucuronides based on aglycons other than quercetin. Generally, the fragmentation pattern displayed by these complexes may only be said to be indicative of B-ring glucuronidation, without specifying the particular position of conjugation.

A third type of complex, [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$, provides results similar to those obtained from [Co(II) (FG-H) (4,7-dmphen)$_2$]$^+$, but is worth discussion because this complex was found to be the most adaptable to LC-MS

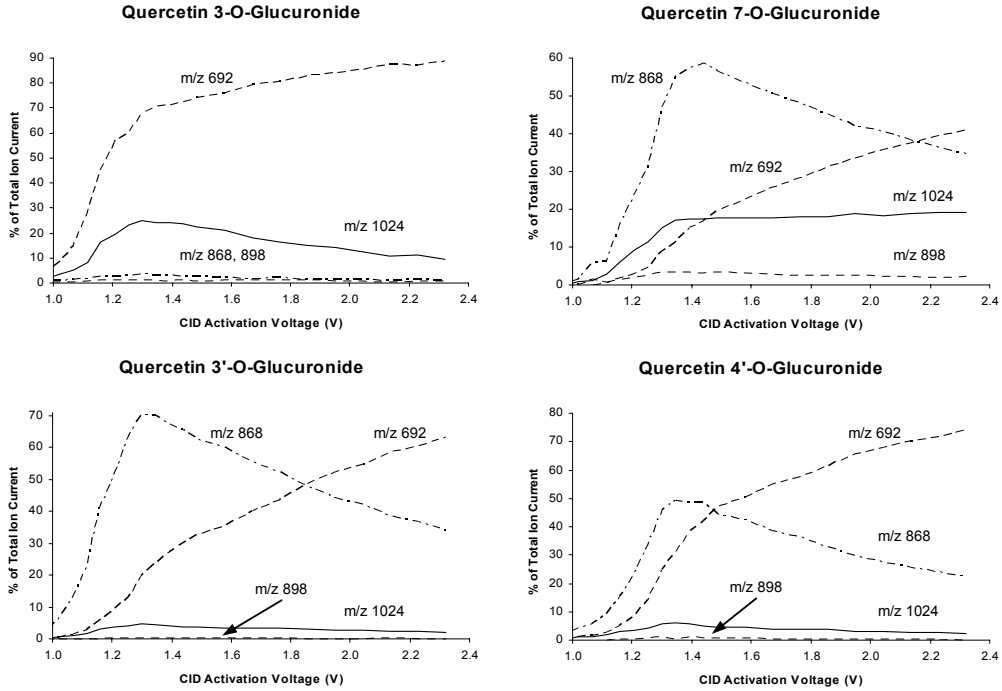


Figure 6.6. Energy variable CID results from [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ complexes involving quercetin glucuronides. The precursor ion and minor product ions are omitted. Fragments are identified as: m/z 1024 (loss of the glucuronic acid moiety), m/z 898 (loss of the aglycon portion), m/z 868 (loss of 4,7-dpphen), m/z 692 (loss of 4,7-dpphen and the glucuronic acid moiety).

analysis. The results of the energy variable CID experiments are shown in Figure 6.6. It is seen that the [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ complex involving quercetin 7-O-glucuronide provides a unique signature of product ions (among the quercetin-based complexes), including significant losses of the glucuronic acid moiety and the 4,7-dpphen ligand, both individually and concurrently, as well as loss of the flavonoid aglycon. Naringenin 7-O-glucuronide and baicalin also share these distinctive fragmentation characteristics (not shown). Quercetin 3-O-glucuronide can be differentiated by the lack of a highly abundant loss of 4,7-

dpphen. Two other compounds, quercetin 3′-O-glucuronide and quercetin 4′-O-glucuronide, are differentiated based on relative ion abundances. The two most abundant products, the ions of m/z 692 and m/z 868, correspond to the loss of a 4,7-dpphen molecule either with or without the glucuronic acid moiety. In the range of 1.5-1.8 V of activation potential, the ion of m/z 868 is consistently the more abundant fragment of the quercetin 3′-O-glucuronide complex, while the ion of m/z 692 is the more abundant fragment of the quercetin 4′-O-glucuronide complex. Therefore these two isomers can be confidently differentiated using a CID voltage in the range of 1.5-1.8 V. As with the other complexation modes, the differentiation of 3′-O-glucuronide from 4′-O-glucuronide applies only to quercetin derivatives and is not expected to be effective for derivatives of other flavonoids.

6.3.2 Spiked Rat Plasma

The concentration of flavonoid glucuronides in human blood is typically between 0.1 to 2 μM following the consumption of flavonoid-rich foods.[6,28] Experiments were performed to determine whether metal complexation methods are effective at these low concentrations. A solution of the four quercetin glucuronide standards (1 μM each) was prepared and analyzed by LC-MS^n with post-column complexation. Each compound could be identified based on the fragmentation of $[Co(II)\ (FG - H)\ (4,7\text{-dpphen})_2]^+$ (m/z 1200).

Having established that 1 μM concentrations can be analyzed in this way, the quercetin glucuronides were spiked into rat plasma at the 1 μM level, and were then extracted and analyzed as described in Section 6.2.3. All four compounds could be observed and identified in this manner. Based on the similarities between these MS/MS results and those obtained by direct infusion, the four compounds were identified as: quercetin 7-O-glucuronide (retention time 10.5 min), quercetin 3-O-glucuronide (retention time 14.1 min), quercetin 4′-O-glucuronide, (retention time 17.4 min), and quercetin 3′-O-glucuronide (retention time 20.5 min). These results are promising in terms of using LC-MS methods to identify flavonoid glucuronides from blood samples obtained from *in vivo* studies.

6.3.3 Grapefruit Juice Study

Urine samples were obtained from a metabolism study involving the consumption of grapefruit juice.[17] In that study, several glucuronidated and sulfated metabolites were partially identified by LC-MS on the basis of characteristic losses from the metabolites. However, the positions of glucuronidation could not be determined conclusively. One of these urine samples was re-analyzed using the new methods reported herein. The sample was prepared and analyzed as described in Section 6.2.4. Two naringenin glucuronides were found using LC-MS in the negative ESI mode by the m/z value

of the deprotonated analyte (m/z 447) and the characteristic loss of the glucuronic acid moiety (-176 Da) upon dissociation.

When post-column complexation was employed, the analytes formed ample complexes of the type [Co(II) (FG - H) (4,7-dpphen)$_2$]$^+$ of m/z 1170. Identification of the glucuronidation position was made by comparing the MS/MS data (Figure 6.7) to those collected by direct infusion as described above. The fragmentation behavior of complex **1** matched that of the naringenin 7-O-glucuronide complex. This assignment is consistent with the results of an alternate identification method based on retention time matching with the

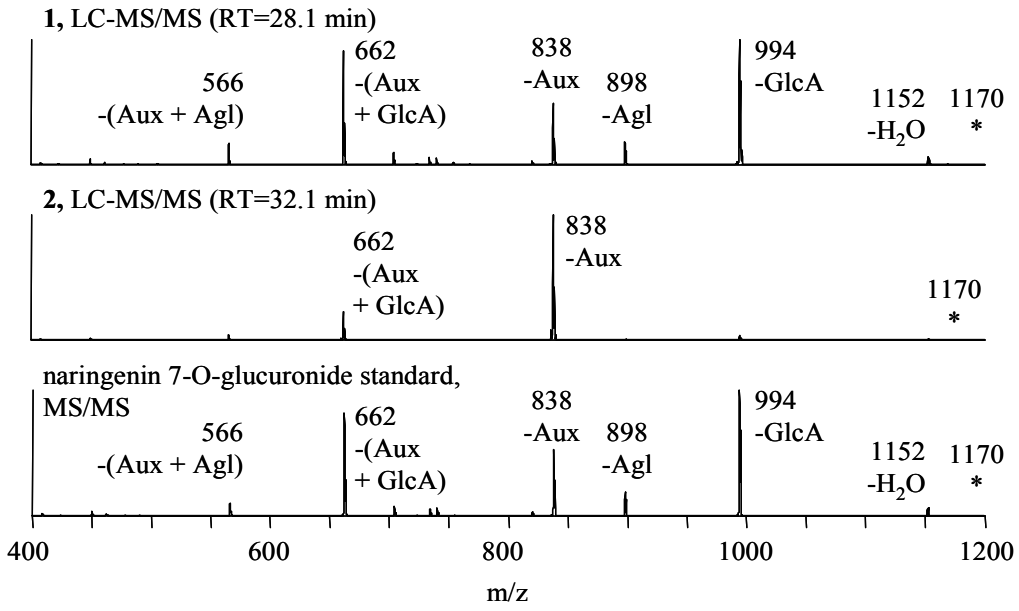


Figure 6.7. LC-MS/MS spectra of [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ (m/z 1170) from human urine following consumption of grapefruit juice. A collision energy of 1.59 V was used. The direct infusion MS/MS spectrum of the same complex involving a standard of naringenin 7-O-glucuronide is also shown.

authenticated standard from the Institute of Food Research. Complex **2** represents the more typical case where an authenticated standard is not available and the dissociation behavior of the complex is not known beforehand. This complex yields two significant product ions corresponding to the loss of 4,7-dpphen with and without the glucuronide moiety. This mirrors the behavior of the quercetin 3′-O-glucuronide and quercetin 4′-O-glucuronide complexes. As naringenin does not possess a hydroxyl group at the 3′ position, **2** was assigned as naringenin 4′-O-glucuronide. This assignment is in agreement with Abe et al., who noted that naringenin 4′-O-glucuronide is retained longer by reversed-phase chromatography than the analogous 7-O-glucuronide.[38] The similarity of the fragmentation pathways suggests that consistent fragments found for model compounds may be extended to identify other flavonoid glucuronides without standards.

6.3.4 Orange Intervention Study

Urine samples from nineteen volunteers in an orange intervention study were screened for flavonoid metabolites. Five metabolites (**3**-**7**) were identified at the Institute of Food Research and were independently and blindly confirmed using metal complexation methods. Negative ion mode analysis of components **3** (retention time = 11.7 min) and **4** (RT = 12.9 min) revealed the masses of both of these compounds to be 448 Da. In each case, CID led to the loss of 176 Da,

corresponding to a glucuronic acid moiety and producing a fragment ion of m/z 271. The second-generation fragment ions stemming from the aglycon portion (m/z 271) matched the fragmentation pattern of deprotonated naringenin, a member of the flavanone group whose glycosides are known to occur in oranges.[39] Post-column metal complexation was then performed to pinpoint the specific sites of glucuronidation of the two metabolites. The fragmentation of the metal complexes of **3** and **4** were very similar to those of **1** and **2** from the grapefruit juice study. Using the same reasoning as before, **3** was identified as naringenin 7-O-glucuronide and **4** as naringenin 4′-O-glucuronide.

Similar evidence was used to identify unknowns **5** (RT = 14.9 min) and **6** (RT = 17.8 min). Negative ion mode LC-MS revealed two compounds of mass 478 Da (based on the formation of deprotonated molecular ions of m/z 477) that fragmented to yield a single neutral loss of 176 Da, corresponding to the glucuronic acid moiety. The second-generation fragment ions from subsequent MS^3 experiments are indicative of hesperetin as revealed based on comparison to the fragmentation pattern of a standard (Figure 6.8). Fragmentation of the metal complex of **5** indicates a glucuronic acid moiety at the 7 position, whereas the metal complex of **6** indicates B-ring glucuronidation, i.e. at the 3′ position of hesperetin (Figure 6.9). Thus **5** was positively identified as hesperetin 7-O-glucuronide and **6** as hesperetin 3′-O-glucuronide. **7** was partially identified as a hesperetin sulfate based on its mass (382 Da), the loss of 80 Da upon

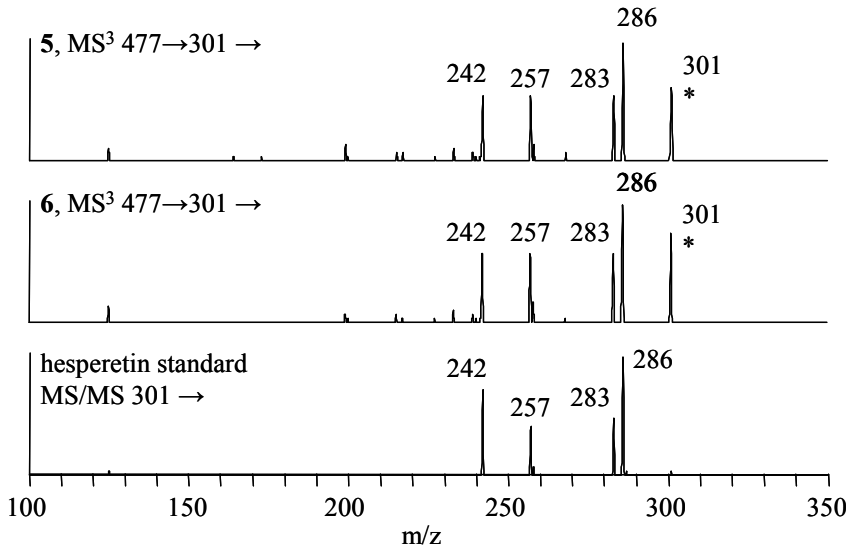


Figure 6.8. Negative ion mode LC-MS^3 spectra used to identify flavonoid glucuronides in urine samples from the orange intervention study. A direct infusion MS/MS spectrum of a hesperetin standard is shown for comparison.

fragmentation (corresponding to SO_3), and second-generation fragment ions that resemble those of hesperetin. To the best of my knowledge, there is no purely mass spectrometry-based method for determining the location of sulfate groups on flavonoids.

In addition to the above compounds, the LC-MS^n analysis additionally revealed several disubstituted metabolites in the urine samples. A hesperetin glucuronide sulfate (RT = 16.6 min) was identified based on its mass (558 Da), neutral losses of 80 Da and 176 Da upon CID, and an aglycon fragmentation signature matching that of hesperetin. Surprisingly, metal complexation was able to reveal the location of the glucuronic acid moiety of this compound. The metal

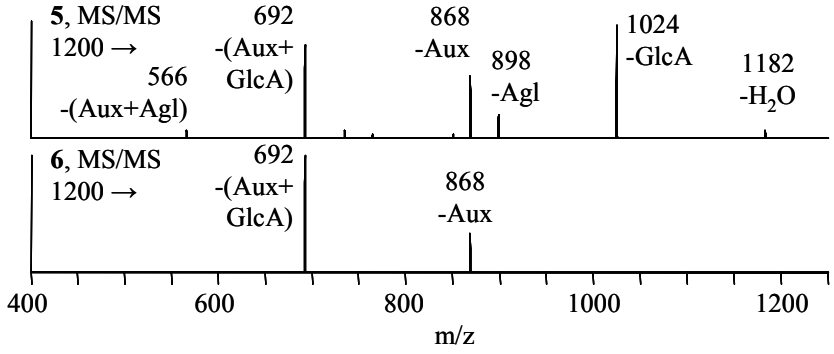


Figure 6.9. LC-MS/MS spectra of [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ (m/z 1200) of hesperetin glucuronides in urine samples from the orange intervention study.

complexation technique was developed originally for the characterization of flavonoid monoglucuronides, but the hesperetin glucuronide sulfate also formed a metal complex of the correct stoichiometry, observed at m/z 1280 in the positive mode ESI mass spectrum. The first stage of CID led to the loss of the sulfate group (-80 Da), effectively resulting in a metal complex of a flavonoid monoglucuronide, which was then characterized based on its fragmentation pattern as described for the other compounds above. The second stage of fragmentation, using m/z 1200 as the precursor ion, yielded a CID mass spectrum similar to that shown in Figure 6.9 for unknown **5**. This strongly suggests that the glucuronic acid moiety of this hesperetin glucuronide sulfate is located at the 7 position. A similar strategy was used previously to partially identify a diglycosyl flavonoid from an onion extract while employing metal complexes designed to characterize monoglycosyl flavonoids (discussed in Section 5.3.3). Finally, a few

diglucuronidated flavonoids were found in the urine samples. Two early-eluting components (RT = 4.8 and 9.9 min) were identified as hesperetin diglucuronides. These analytes both had a mass of 654 Da, exhibited two sequential losses of glucuronic acid (-176 Da), and yielded fragmentation patterns of the aglycon group that corresponded to hesperetin. In addition there was at least one other low-intensity compound resembling a naringenin diglucuronide that appeared in only a few urine samples. This analyte had a mass of 624 Da, had a retention time of 1.9 minutes, and lost two glucuronic acid moieties upon sequential stages of CID. However, the low abundance of this analyte combined with signal losses due to scattering during each stage of fragmentation led to a MS^4 spectrum with a poor signal-to-noise ratio. While there was evidence of naringenin aglycon, the spectrum was too noisy to make a conclusive identification. Metal complexes could not be formed for any of the purported naringenin or hesperetin diglucuronides, possibly due to low abundance of these compounds.

6.3.5 Cell Culture Extracts

Two cell culture samples were prepared, one treated with galangin and the other with kaempferol. Full scan mass chromatograms were obtained from each sample, and the data was subsequently examined for the presence of likely galangin and kaempferol metabolites: glucuronides, sulfates, and combinations thereof. The first cell culture sample showed, in addition to unreacted galangin,

three potential monoglucuronides **8**-**10** (m/z 445) and one potential sulfate **11** (m/z 349). There was no evidence of doubly-substituted galangin in the sample.

Multiple-stage tandem mass spectrometry was used to check these tentative identifications. A fragmentation spectrum of the galangin aglycon in the sample was taken for comparative purposes. In negative ion mode, each of the three suspected galangin glucuronides lost 176 Da upon CID, confirming the presence of glucuronic acid in their chemical structures. Likewise, the purported galangin sulfate lost the correct mass of 80 Da upon fragmentation, corresponding to a sulfate group. The second-generation fragmentation ion patterns were used to check the structure of the aglycon portion of the molecule. The four suspected galangin conjugates all yielded second-generation fragment ion spectra that are similar to that of galangin aglycon. Postcolumn metal complexation was performed as described in the Experimental section to determine the positions of the glucuronic acid moieties of compounds **8**-**10** (Figure 6.10). The metal complex of **9** gave the most distinctive fragmentation pattern, clearly identifying **9** as a 7-O-glucuronide based on the unique losses of the aglycon portion with and without an auxiliary ligand. Such losses only occur for flavonoid 7-O-glucuronides. Besides the 7 position, galangin has two other positions occupied by hydroxyl groups that might be conjugated with glucuronic acid: the 3 position and the 5 position (see Figure 1.2). The presence of three galangin monoglucuronides in the sample suggests that the conjugation products of all

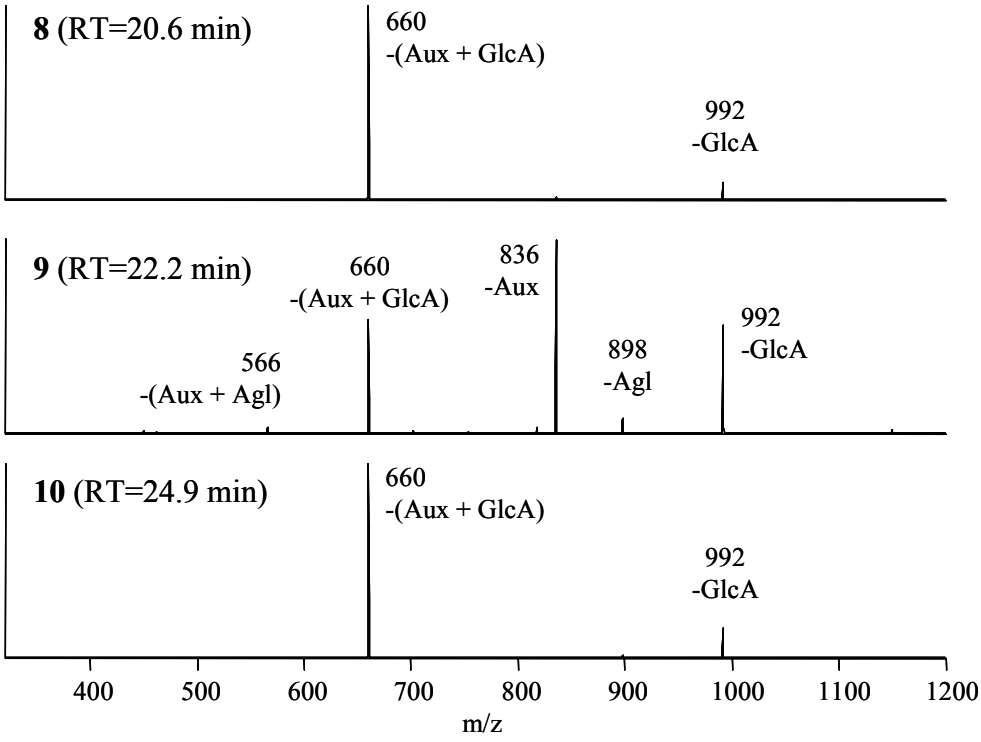


Figure 6.10. LC-MS/MS spectra of [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ flavonoid glucuronide complexes (m/z 1168) from cell cultures treated with galangin.

three hydroxylation positions are present. Yet the metal complexation fragments of **8** and **10** are nearly identical. The fragmentation pattern is apparently indicative of 3-O-glucuronidation based on the absence of a fragment ion corresponding to the loss of an auxiliary ligand. This trait was observed from complexes of quercetin 3-O-glucuronide, but not from any of its isomers (see Figure 6.6). Yet either **8** or **10** must be galangin 5-O-glucuronide. In order to decide which is the 3-O-glucuronide and which is the 5-O-glucuronide, a search through the literature was conducted in order to determine the relative elution

order of 3-O- and 5-O-glycosides of flavonoids. Grayer et al. showed that luteolin 5-O-glucoside elutes slightly before luteolin 7-O-glucoside, and apigenin 5-O-glucoside elutes slightly before apigenin 7-O-glucoside in reversed-phase chromatography.[40] Similarly, Harborne and Boardley demonstrated that quercetin 5-O-glucoside elutes before quercetin 3-O-glucoside using reversed-phase HPLC.[41] Finally, the spiked rat plasma sample showed that quercetin 7-O-glucuronide elutes before quercetin 3-O-glucuronide (described in Section 6.3.2). Taken together, along with the general observation that similar types of elution order trends have been found for flavonoid and their glycosides, these examples imply that for flavonoid glycoside isomers having the same parent aglycon and saccharide but differing only by position of conjugation, the generalized order of elution is 5-O-glycoside, 7-O-glycoside, 3-O-glycoside. Thus this is the proposed elution order of the three galangin glucuronides, and it fits with the metal complexation data. The identical fragmentation of the 3-O-glucuronide and 5-O-glucuronide complexes suggests that these positions may not be differentiated only on the basis of the $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ fragmentation patterns.

In the cell culture sample that had been treated with kaempferol, there appeared two potential kaempferol glucuronides (**12** and **13**) and one potential kaempferol sulfate (**14**), as determined by mass. Again, there was no evidence of any doubly-substituted conjugates. The loss of a glucuronic acid moiety (-176 Da) or a sulfate group (-80 Da) was confirmed in the first stage of CID.

Confirming the aglycon structure was more difficult. In contrast to most other cases, the MS^3 fragmentation of kaempferol derivatives sometimes fails to closely resemble the MS/MS fragmentation of native kaempferol aglycon using negative ion mode analysis. This unusual phenomenon will be explored more thoroughly in Chapter 8. The MS^3 spectra of the metabolites in the cell culture showed only a subset of the numerous peaks yielded by fragmenting a standard of native kaempferol aglycon. However, this was enough to confirm that the glucuronidated and sulfated conjugates were indeed kaempferol derivatives.

The two glucuronidated compounds were subjected to postcolumn metal complexation for further structural analysis. The metal complex of **12** yielded the characteristic array of fragment ions denoting a 7-O-glucuronide, including the

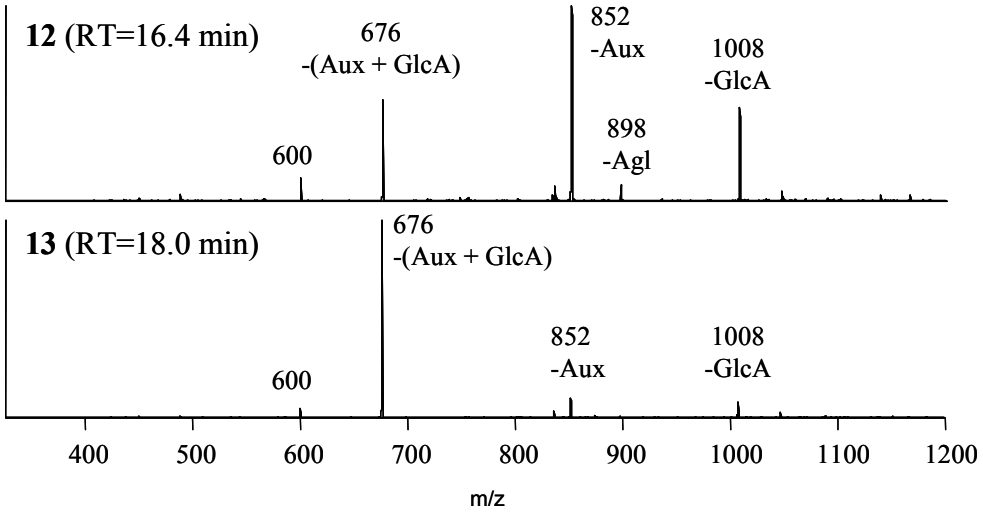


Figure 6.11. LC-MS/MS spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ flavonoid glucuronide complexes (m/z 1184) from cell cultures treated with kaempferol. M/z 600 is a background peak.

loss of the aglycon portion of the molecule (Figure 6.11). The metal complex of **13** fragmented into a minor product corresponding to the loss of an auxiliary ligand and a major product corresponding to the loss of an auxiliary ligand with a glucuronic acid moiety. This pattern typically denotes a 3-O-glucuronide, but the galangin cell culture example shows that the same pattern may be indicative of 5-O-glucuronides as well. Once again, the evidence of retention time is used to settle the question. As unknown **13** elutes after kaempferol 7-O-glucuronide, it is assigned as kaempferol 3-O-glucuronide rather than kaempferol 5-O-glucuronide.

6.4 CONCLUSIONS

A versatile method for differentiating isomeric flavonoid glucuronides by mass spectrometry has been discovered. Complexes are formed by mixing a flavonoid glucuronide, $CoBr_2$ and an auxiliary ligand (either 4,7-dmphen or 4,7-dpphen) in solution. Performing ESI-MS/MS on these complexes leads to characteristic product ions that allow isomer differentiation and determination of the position of the glucuronide moiety. Four isomeric quercetin glucuronides were differentiated based on unique product ion profiles obtained from these complexes. Glucuronide derivatives of kaempferol, naringenin and baicalein formed similar complexes which fragmented in an analogous manner to the quercetin glucuronides, allowing the location of the glucuronide moiety to also be determined for these compounds. These examples suggested the possibility that

glucuronide derivatives of other flavonols, flavones and flavanones may be characterized in the same way. This method may be performed by direct infusion ESI-MS/MS (using pure flavonoid glucuronides) or by LC-MS/MS of mixtures using post-column complexation. The method was also proven to be sensitive enough for *in vivo* plasma analysis at realistic analyte concentrations. The LC-MS/MS method was used to support the identification of naringenin 7-O-glucuronide and naringenin 4′-O-glucuronide in human urine following the consumption of grapefruit juice. The same compounds were found in human urine after consumption of orange juice, as well as hesperetin 7-O-glucuronide, hesperetin 3′-O-glucuronide, and a hesperetin glucuronide sulfate with the glucuronic acid moiety at the 7 position. The identities of the metabolites from the orange intervention study were confirmed independently at the Institute of Food Research. Glucuronidated derivatives of galangin and kaempferol were also identified in cell cultures. Evidence suggests that 5-O-glucuronidation and 3-O-glucuronidation may be indistinguishable by the current method, but retention time analysis may help resolve ambiguous cases. Overall, metal complexation has proven to be a successful and widely applicable approach to determining the glucuronidation position of flavonoids.

6.5 REFERENCES

(1) Manach C, Donovan JL. *Free Radical Research* **2004**, *38*, 771-785.
(2) Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G, Johnson IT. *Journal of Nutrition* **2000**, *130*, 2765-2771.
(3) Williamson G, Day AJ, Plumb GW, Couteau D. *Biochemical Society Transactions* **2000**, *28*, 16-22.
(4) Day AJ, Williamson G. *British Journal of Nutrition* **2001**, *86*, S105-S110.
(5) Sesink ALA, O'Leary KA, Hollman PCH. *Journal of Nutrition* **2001**, *131*, 1938-1941.
(6) Williamson G, Barron D, Shimoi K, Terao J. *Free Radical Research* **2005**, *39*, 457-469.
(7) Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G. *American Journal of Clinical Nutrition* **2004**, *80*, 15-21.
(8) Williamson G. *Phytochemistry Reviews* **2002**, *1*, 215-222.
(9) Roseiro LB, Viala D, Besle JM, Carnat A, Fraisse D, Chezal JM, Lamaison JL. *International Dairy Journal* **2005**, *15*, 579-584.
(10) Edenharder R, Keller G, Platt KL, Unger KK. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2767-2773.
(11) Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, **1970**.
(12) Day AJ, Bao YP, Morgan MRA, Williamson G. *Free Radical Biology and Medicine* **2000**, *29*, 1234-1243.
(13) Franski R, Eitner K, Sikorska M, Matlawska I, Stobiecki M. *Phytochemical Analysis* **2003**, *14*, 170-175.
(14) Wittig J, Herderich M, Graefe EU, Veit M. *Journal of Chromatography B* **2001**, *753*, 237-243.
(15) Mullen W, Boitier A, Stewart AJ, Crozier A. *Journal of Chromatography A* **2004**, *1058*, 163-168.
(16) Matsumoto H, Ikoma Y, Sugiura M, Yano M, Hasegawa Y. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 6653-6659.
(17) Zhang JM, Brodbelt JS. *Analyst* **2004**, *129*, 1227-1233.
(18) Murota K, Mitsukuni Y, Ichikawa M, Tsushida T, Miyamoto S, Terao J. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 1907-1912.
(19) Arts ICW, Sesink ALA, Faassen-Peters M, Hollman PCH. *British Journal of Nutrition* **2004**, *91*, 841-847.
(20) Needs PW, Kroon PA. *Tetrahedron* **2006**, *62*, 6862-6868.
(21) O'Leary KA, Day AJ, Needs PW, Sly WS, O'Brien NM, Williamson G. *FEBS Letters* **2001**, *503*, 103-106.
(22) Davis BD, Needs PW, Kroon PA, Brodbelt JS. *Journal of Mass Spectrometry* **2006**, *41*, 911-920.

(23) Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.
(24) McClellan JE, Murphy JP, Mulholland JJ, Yost RA. *Analytical Chemistry* **2002**, *74*, 402-412.
(25) Vrkic AK, O'Hair RAJ. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 715-724.
(26) Jellen EE, Chappell AM, Ryzhov V. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 1799-1804.
(27) Vaisar T, Gatlin CL, Rao RD, Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2001**, *36*, 306-316.
(28) Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G. *Free Radical Research* **2001**, *35*, 941-952.
(29) Domon B, Costello CE. *Glycoconjugate Journal* **1988**, *5*, 397-409.
(30) Satterfield M, Brodbelt JS. *Analytical Chemistry* **2000**, *72*, 5898-5906.
(31) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(32) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(33) Zhang JM, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1761-1770.
(34) Zhang JM, Wang JM, Brodbelt JS. *Journal of Mass Spectrometry* **2005**, *40*, 350-363.
(35) Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
(36) Davis BD, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1883-1890.
(37) Clowers BH, Hill Jr. HH. *Journal of Mass Spectrometry* **2006**, *41*, 339-351.
(38) Abe K, Katayama H, Suzuki A, Yumioka E. *Shoyakugaku Zasshi* **1993**, *47*, 402-407.
(39) Cuthrell K, Le Marchand L. In *Potential Health Benefits of Citrus*; Patil BS, Turner ND, Miller EG, Brodbelt JS, Ed.; American Chemical Society: Washington DC, **2006**.
(40) Grayer RJ, Kite GC, Veitch NC, Eckert MR, Marin PD, Senanayake P, Paton AJ. *Biochemical Systematics and Ecology* **2002**, *30*, 327-342.
(41) Harborne JB, Boardley M. *Journal of Chromatography A* **1984**, *299*, 377-385.

Regioselectivity of Human UDP-Glucuronosyltransferase 1A1 in the Synthesis of Flavonoid Glucuronides Determined by Metal Complexation and Tandem Mass Spectrometry

7.1 INTRODUCTION

Much progress has been made in the understanding of flavonoid metabolism over the past decade. The currently-accepted paradigm involves the consumption of flavonoid glycosides in plant-based food products, deglucosylation in the small intestine by β-glucosidase or lactose phloridzin hydrolase, and rapid metabolism by Phase I and (especially) Phase II enzymes.[1-3] Glucuronidation and sulfation are important metabolic routes for most flavonoids, while methylation or hydroxylation may also occur depending on the structure of the molecule in question.[2,3] There has also been a report of glutathione-related metabolites in human urine[4] As a result of these rapid conjugation reactions, neither the original flavonoid glycosides (except anthocyanins) nor the aglycon forms (except catechins) are found in plasma.[5-8] Early reports of unmodified flavonoid glycosides circulating in the bloodstream[9-11] were likely mistaken identifications of flavonoid glucuronides, which have similar chromatographic and ultraviolet (UV) spectroscopic characteristics.[7,12] Flavonoids that fail to be absorbed in the small intestine may be broken down by microflora in the large intestine.[1-3] This process may release the free aglycons, which can then be absorbed and metabolized, but mostly results in the release of small phenolic

acids, which are expelled in the urine.[1-3] Quantitative *in vivo* studies generally show that only a small percentage of consumed flavonoid glycosides is recovered in urine as conjugated phase II metabolites.[13-15] Walle et al. used ^{14}C-labelled quercetin to show that up to 81% of the administered dose ultimately is exhaled in the form of carbon dioxide.[16] There remains considerable interest in the conjugated metabolites as they may retain some of the bioactivity of the original molecules.[5,17]

In spite of breakthroughs in the field of flavonoid metabolism, much is still unknown about the precise structure of Phase II flavonoid metabolites, particularly in terms of the conjugation positions. Most reports do not supply this information, identifying observed metabolites imprecisely as, for example, unspecified quercetin glucuronides. A recent review[5] listed all conjugated metabolites that have been identified in human *in vivo* studies, but the very short list included conjugates of only a few flavonoid aglycons. One reason for the dearth of such information is a lack of sensitive and specific analytical methods. While there are several approaches currently available, there are problems associated with each. The standard method for structural determination of organic molecules is nuclear magnetic resonance (NMR) spectroscopy, but its lack of sensitivity does not lend itself to analysis of low-concentration metabolites. Thus few metabolism studies have employed this technique.[18,19] An alternative method using UV spectroscopy[20] to determine the conjugation position of flavonoids has

been used only rarely for metabolites,[17] possibly due to the complex set of experiments with numerous UV shift reagents required to make this determination. A third strategy requires synthesis of suspected metabolites and comparison of the chemical properties of these synthetic compounds to those of the observed metabolites. While this approach is sometimes employed to identify flavonoid metabolites,[13,21,22] it is too laborious for routine use.

Chapter 6 gave details on a metal complexation/tandem mass spectrometry method for differentiating the conjugation positions of monoglucuronidated flavonols, flavones and flavanones.[31] The metal complexation strategy entailed formation of complexes of the type $[Co(II)\ (FG - H)\ (4,7\text{-dpphen})_2]^+$ where (FG - H) is one molecule of deprotonated flavonoid glucuronide and $(4,7\text{-dpphen})_2$ are two molecules of an auxiliary ligand, 4,7-diphenyl-1,10-phenanthroline. Upon CID, this complex yielded product ions corresponding to the loss of a glucuronic acid moiety, of an auxiliary ligand, and of the aglycon portion of the flavonoid, in various combinations and ratios that correlated with the position of glucuronidation. A four-way differentiation of the 3-O-, 7-O-, 3′-O- and 4′-O-glucuronides of quercetin was demonstrated. This previous investigation focused on a limited set of flavonoid glucuronides, but it was speculated that the versatility and sensitivity of the method would allow facile adaptation to other flavonoid metabolites, such as the ones produced enzymatically in the present study.

The purpose of the current work is twofold. In order to add to the growing body of knowledge regarding flavonoid metabolism, the regiospecificity of flavonoid glucuronidation by the 1A1 isozyme of human UDP-glucuronosyltransferase (UGT1A1) is probed. While there have been several studies on the glucuronidating activity of various isozymes of UGT using flavonoids as substrates,[32-38] fewer have explored the regioselectivity of such reactions.[19,39] The second goal is to explore the wider applicability of the described metal complexation methods to flavonoid metabolism studies. The glucuronidated derivatives of ten flavonols, flavones and flavanones provide a diverse set of analytes to challenge the performance of the new method. The correlation between flavonoid conjugation sites and reversed-phase high-performance liquid chromatography (HPLC) retention time is also discussed and used as an additional tool in elucidating the structures of the enzymatically synthesized products.

7.2 EXPERIMENTAL

7.2.1 Materials

UGT1A1 isozyme (human, recombinant), UDP-glucuronic acid (UDPGA) trisodium salt, 4,7-diphenyl-1,10-phenanthroline (4,7-dpphen), cobalt(II) bromide, acacetin, myricetin, hesperetin, naringenin and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). Eriodictyol, kaempferol, luteolin, luteolin

7-O-glucoside and luteolin 4′-O-glucoside were purchased from Indofine (Hillsborough, NJ). Apigenin, homoeriodictyol, quercetin 3-O-glucoside and quercetin 4′-O-glucoside were purchased from Extrasynthèse (Genay, France). Quercetin 7-O-glucoside was purchased from Apin (Abingdon, UK). Quercetin 3-O-glucuronide, quercetin 7-O-glucuronide, quercetin 3′-O-glucuronide and quercetin 4′-O-glucuronide were synthesized [22,31] by Paul Needs and Paul Kroon at the Institute of Food Research (Norwich, UK). Potassium phosphate was purchased from Thermo Fisher Scientific, Inc. (Waltham, MA).

7.2.2 Enzymatic Synthesis of Flavonoid Glucuronides

The method for synthesizing the flavonoid glucuronides was adapted from one described by Plumb et al.[40] The UGT1A1 enzyme, having an estimated concentration of 12 mg/mL, was divided into 25 μL aliquots which were stored at -80 °C until use. Flavonoids were prepared as 10 mM solutions in methanol, which were employed despite turbidity of a few solutions. Synthesis occurred in a microcentrifuge tube, to which were added 2 mM aqueous UDPGA (65 μL), 20 mM potassium phosphate buffer pH 7.0 (378.75 μL) and 10 mM flavonoid (6.25 μL). The reaction was initiated by adding a 25 μL aliquot of UGT1A1 to the tube. The tubes were incubated in a 37 °C water bath (VWR Model 1227, West Chester, PA) with gentle agitation for 6 hours. A few reactions were repeated with 1 hour incubation times, but these gave similar results to the 6 hour

incubations, so these data will not be specifically presented here. Reactions were stopped by adding 1.5 mL acetone. The tubes were centrifuged for 10 minutes at 16000 g, and the supernatants were removed and blown with nitrogen to evaporate the acetone. The remaining supernatant was refrigerated until analysis.

7.2.3 LC-MS Conditions

Liquid chromatography took place on a Waters Alliance 2695 HPLC system (Milford, MA). The stationary phase was a Waters Symmetry C18 column, 2.1 x 50 mm, 3.5 μm particle size, with a guard column. Typical injection volumes were 15 to 30 μL for negative ion mode analyses, and 30 to 45 μL for positive ion mode. The mobile phases were 0.33% formic acid in water (A) and 0.33% formic acid in methanol (B). A typical gradient began at 35% B, increased to 50% B over 25 minutes, then increased to 95% B over 2 minutes, with a constant flow rate of 0.1 mL/min. For the naringenin, eriodictyol and homoeriodictyol reaction products, the first part of the gradient increased from 30% B to 45% B in 25 minutes; while for acacetin the first part of the gradient increased from 40% B to 55% B in 25 minutes. The column effluent was sent directly to the mass spectrometer.

All mass spectrometry was performed on an LCQ Duo (Thermo Electron, Waltham, MA) quadrupole ion trap with electrospray ionization (ESI). Samples were analyzed in both negative and positive ion modes. In both polarities, a spray

voltage of 5.0 kV was used, the heated capillary temperature was 200 °C, and the automatic gain control was set to a target of 2 x 10^7 ions with a maximum injection time of 500 msec and 5 microscan averaging. All other mass spectrometer parameters were set as needed to obtain optimal signal. Negative ion mode was used to search for flavonoid glucuronides and to confirm the identities of the aglycon portion of these molecules. Positive ion mode was used for analysis of metal complexes, which were formed by post-column addition of a methanolic solution of 5 μM $CoBr_2$ and 4,7-dpphen. These reagents were infused at a rate of 20 μL/min controlled by a syringe pump, and added to the column effluent via a tee between the column and the mass spectrometer. Due to the fragility of the metal complexes, an isolation width of 4 Da was used.[41] A constant normalized collision energy[42] of 35% (of 5 V_{p-p}) was used to dissociate the complexes.

7.2.4 HPLC Retention Time Analysis

Retention time was used as a source of supplementary data for the identification of the flavonoid glucuronides. In order to determine trends in retention order, flavonoid glycoside standards were injected individually, and the retention times were measured by a Waters 486 UV detector. All other chromatographic equipment was the same as previously described. The gradient

employed began at 15% B, increased to 70% B over 12 minutes, then increased to 95% B over 2 minutes, with a flow rate of 0.3 mL/min.

7.3 RESULTS AND DISCUSSION

7.3.1 Strategy

For identification of the flavonoid glucuronides, the two key steps include the determination of the aglycon skeleton and the location of the site of conjugation. In our approach, the aglycon skeleton is elucidated from MS^n spectra of the deprotonated flavonoid glucuronide with spectral comparison to standard flavonoid aglycons. The site of conjugation is pinpointed based on interpretation of the MS/MS spectra of metal complexes of the type [Co(II) (FG - H) $(4,7\text{-dpphen})_2]^+$. As discussed in Chapter 6, the characteristic signs of 7-O-glucuronidation from this type of metal complex include significant losses of the glucuronic acid moiety (-GlcA) and of the auxiliary ligand (-Aux), both individually and concurrently, as well as the loss of the flavonoid aglycon (-Agl), which is a diagnostic fragment for the 7-O-glucuronidation position. The metal complexes of B-ring-glucuronides typically display significant amounts of the –Aux and –(Aux + GlcA) fragment ions upon CID, but yield only minor amounts of the -GlcA fragment ion.[31] Upon CID, metal complexes of flavonol 3-O-glucuronides produce the –(Aux + GlcA) fragment ion predominantly.[31]

7.3.2 Single-Product Reactions

After the enzymatic incubation of each of the ten flavonoids, the initial screening employed LC-MS with negative ion mode analysis. The total ion chromatograms were searched for masses corresponding to the aglycon (unreacted starting material), the monoglucuronidated flavonoid (aglycon + 176 Da) and the diglucuronidated flavonoid (aglycon + 176 Da + 176 Da). Unreacted aglycon was observed in most cases, but no diglucuronidated products were produced in any of the reactions. All of the reactions produced monoglucuronidated products, with five flavonoids generating a single product, and the other five producing either two or three products. This section concerns the former group, consisting of apigenin, naringenin, acacetin, kaempferol and homoeriodictyol.

Although most research involving the identification of flavonoid conjugates by mass spectrometry does not employ MS^n for verification of the flavonoid skeleton, a urinary metabolite with the correct mass and initial fragments for a quercetin glucuronide sulfate was recently encountered, but which required MS^4 analysis to prove that it was not a quercetin derivative at all.[43] Hence confirming the identity of the aglycon portion is an important step in identifying these molecules. MS/MS of the all monoglucuronidated products showed the characteristic loss of a glucuronic acid residue, -176 Da. An additional isolation/dissociation step (MS^3) was used to fragment the aglycon

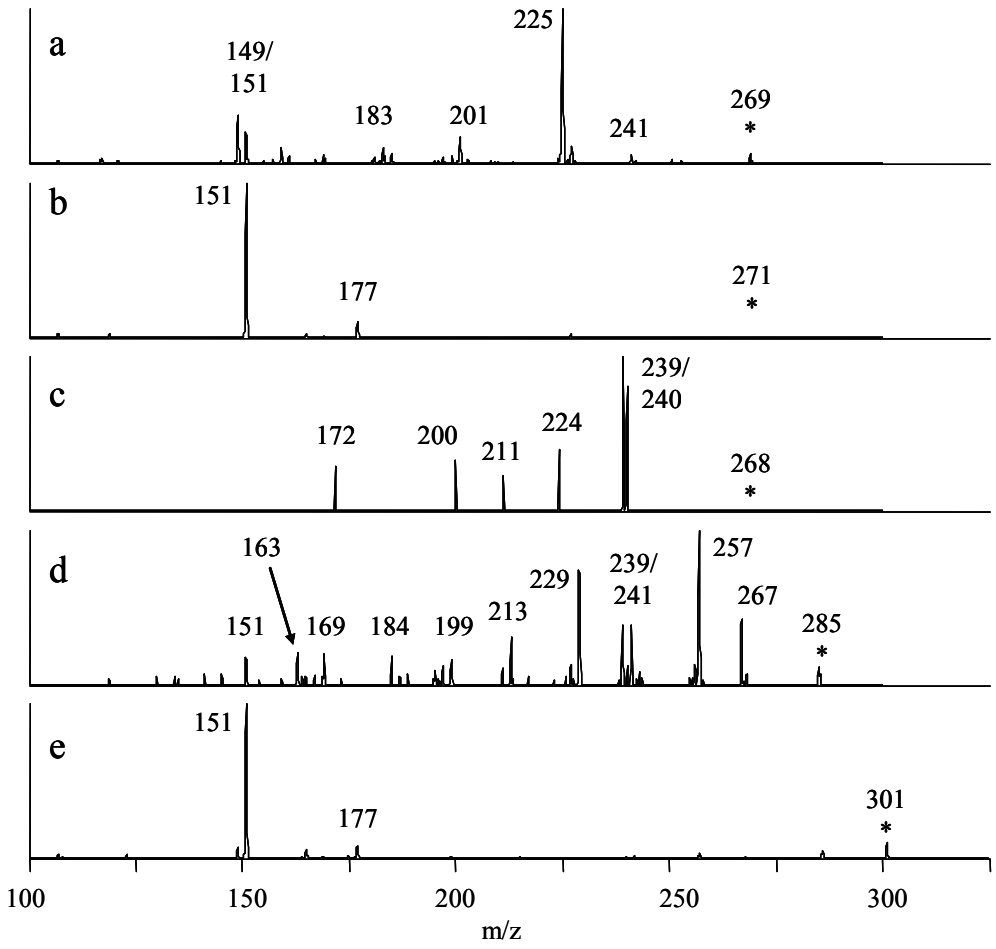


Figure 7.1. MS^n spectra used to confirm the identities of the aglycon skeletons of deprotonated flavonoid monoglucuronides. a) apigenin monoglucuronide, 445→269→ b) naringenin monoglucuronide, 447→271→ c) acacetin monoglucuronide, 459→283→ 268→ d) kaempferol monoglucuronide, 461→285→ e) homoeriodictyol monoglucuronide, 477→301→.

portions of these molecules. The resulting spectra are shown in Figure 7.1. Because the flavonoid aglycons yield a variety of product ions, the structures were easily confirmed by comparing the MS/MS spectra of the aglycon standards,

which were nearly identical to the spectra shown. Acacetin, like many methoxylated flavonoid aglycons, yields only a single fragment ion due to the loss of a methyl radical, which is not a diagnostic dissociation pathway.[44,45] Hence, the MS^4 spectrum of the acacetin monoglucuronide is shown in Figure 7.1 rather than the MS^3 spectrum, and it was compared to the MS^3 spectrum of the acacetin standard. Some of the diagnostic dissociation routes include retro Diels-Alder pathways that lead to the $^{1,3}A^-$ ion (m/z 151) and small molecule losses such as $-H_2O$, -CO and $-CO_2$.[46,47]

The metal complexation methods described in the Experimental section were used to elucidate the location of the glucuronic acid moiety of these monoglucuronidated flavonoids. In particular, complexes of the type $[Co(II)\ (FG - H)\ (4,7\text{-dpphen})_2]^+$ self-assembled in solution, were transported to the gas phase by ESI, and analyzed by tandem mass spectrometry. The CID mass spectra of these complexes are shown in Figure 7.2. All five of the complexes show evidence of glucuronidation at the 7 position, based on the diagnostic pathways summarized above (e.g. -Aux, -GlcA, -Agl), and thus all products are identified as the 7-O-glucuronides of the relevant flavonoid.

The fragmentation patterns also show some dependence on the class of flavonoid in the complex. Complexes involving flavanone (naringenin and homoeriodictyol) 7-O-glucuronides produce greater relative abundances of the diagnostic ion due to loss of the aglycon moiety (-Agl), as well as those due to

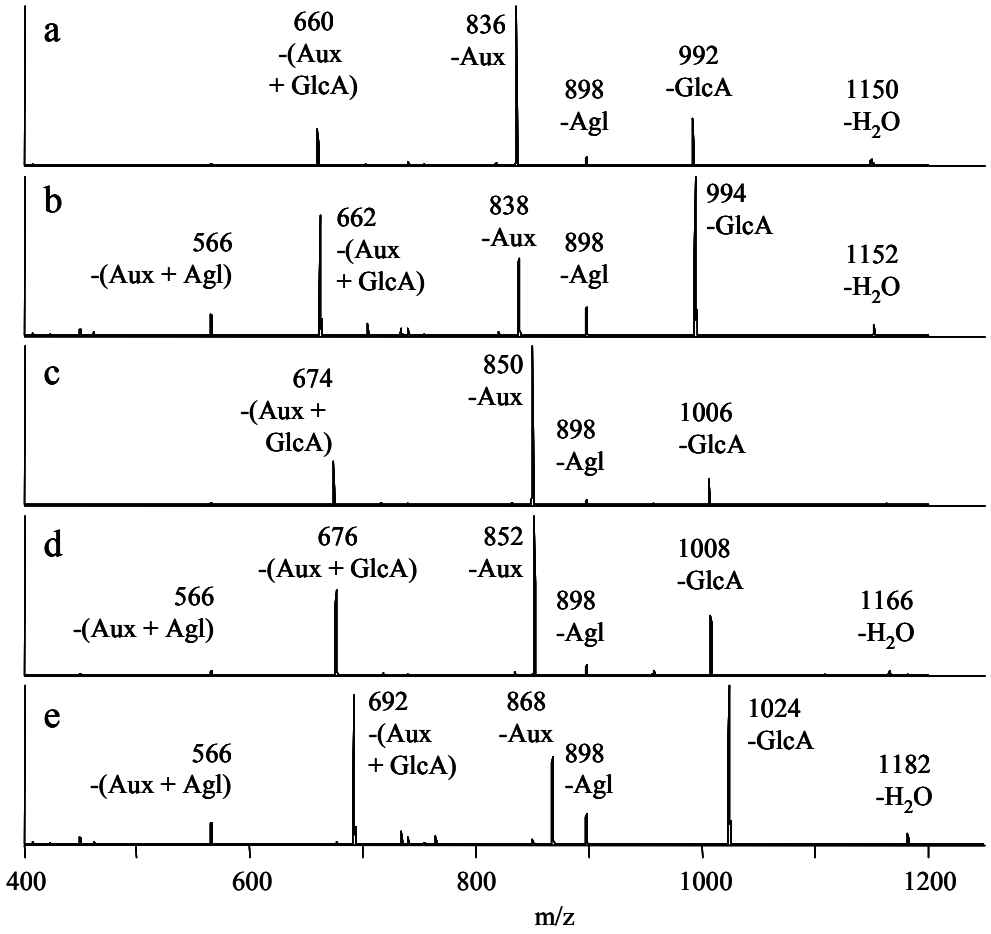


Figure 7.2. CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ complexes of flavonoid monoglucuronides. a) FG=apigenin, 1168→ b) FG=naringenin, 1170→ c) FG=acacetin, 1182→ d) FG=kaempferol, 1184→ e) FG=homoeriodictyol, 1200→.

loss of a glucuronic acid residue (–GlcA) and loss of the auxiliary ligand in conjunction with the elimination of a glucuronic acid residue –(Aux + GlcA). Additionally, they produce a significant fragment ion resulting from the loss of both the auxiliary ligand and the aglycon group, –(Aux + Agl). The relative abundances of these ions are lower for the complex involving the flavonol

(kaempferol) 7-O-glucuronide, and lowest for those complexes involving flavone (apigenin and acacetin) 7-O-glucuronides. The analogous complex involving baicalin (a flavone 7-O-glucuronide) was reported to show similarly low abundances of these fragment ions.[31]

The remaining potential glucuronidation sites should also be considered in case of ambiguous fragment ion signatures from the metal complexes. The –(Agl) and –(GlcA) ions do not feature prominently as dissociation products of flavonoid B-ring-glucuronide complexes, so the glucuronidation at the 3′ and 4′ positions is ruled out. Flavonoid 5-O-conjugates are said to be unfavorable products due to hydrogen bonding to the 4-keto group.[3,47] Nonetheless, an enzymatic synthesis of flavonoid 5-O-glucuronides has been reported.[48,49] Our work has suggested that the fragmentation of metal complexes containing flavonoid 5-O-glucuronides mimics that of the 3-O-glucuronides (see Section 6.3.5). None of the spectra in Figure 7.2 display the –(Aux + GlcA) ion as the only abundant fragment, so both the 3-O- and 5-O-glycosylation positions are ruled out, further corroborating the initial identification of the five unknown compounds as 7-O-glucuronides.

7.3.3 HPLC Retention Time Analysis

The enzymatic incubations of the other five flavonoids (luteolin, eriodictyol, quercetin, hesperetin, and myricetin) all produced more than one

glucuronide derivative. The presence of multiple products means that retention time analysis can be used to assist in compound identification. Since this information plays a crucial role in identifying some of the glucuronidated products, a discussion of this approach is warranted before presenting the remaining mass spectral data.

There are several known trends regarding flavonoid structure and reversed-phase HPLC retention times. Since saccharides are more polar than flavonoid aglycons, flavonoid diglycosides elute before flavonoid monoglycosides, which elute before flavonoid aglycons.[50] It has also been reported that the retention time order is flavanones < flavonols < flavones, for compounds that otherwise have the same substitution patterns.[50] Studies have also shown that the identities of the saccharides influence HPLC mobility in predictable ways. For example, when all else is equal, a 7-rutinoside elutes before a 7-neohesperidoside, and a 3-galactoside elutes before a 3-glucoside.[50] Such trends are upheld in numerous articles which report reversed-phase HPLC retention times, regardless of the exact chromatographic method employed.[30,51-55]

Similar generalizations can be made in cases in which both the flavonoid portion and the glycosidic portion are the same, with only the glycosylation site differing between compounds. Table 7.1 lists the retention times for three groups of such isomers. In all three groups, the 7-O-glycoside elutes before the 4′-O-glycoside. When the 3-O-glycoside is included, it elutes between these two

Table 7.1. Retention time dependence on conjugation position

aglycon	linkage	saccharide	retention time (min)
quercetin	7-O-	glucoside	8.19
quercetin	3-O-	glucoside	9.04
quercetin	4′-O-	glucoside	9.82
luteolin	7-O-	glucoside	7.87
luteolin	4′-O-	glucoside	10.09
quercetin	7-O-	glucuronide	8.72
quercetin	3-O-	glucuronide	9.28
quercetin	4′-O-	glucuronide	10.46
quercetin	3′-O-	glucuronide	11.32

isomers. Finally, quercetin 3′-O-glucuronide elutes after quercetin 4′-O-glucuronide. Thus the retention times of such isomers can be ordered based on glycosylation site as 7 < 3 < 4′ < 3′. This elution order for quercetin monoglucuronides has been reported previously.[12,19,56,57] Regarding the rarer 5-O-glycosides, Grayer et al. reported that the 5-O-glucosides of luteolin and apigenin elute before their 7-O-glucoside analogs.[58] Harborne and Boardley report the retention time of quercetin 5-O-glucoside as lower than that of quercetin 3-O-glucoside.[59] Finally metal complexation has been used to show that the 5-O-glucuronide, the 7-O-glucuronide and the 3-O-glucuronide of galangin elute in that order (Section 6.3.5). The evidence points towards an overall flavonoid glycoside elution order (by position of conjugation) of 5 < 7 < 3 < 4′ < 3′, at least for flavonoid glucosides and glucuronides.

The significance of deciphering this elution rule is that it may be used to confirm the identities of flavonoid glucosides or glucuronides that have been

elucidated by tandem mass spectrometric methods. Moreover, an otherwise unidentifiable isomer may be assigned by retention time if the some of the other isomers have been identified. Both strategies will be applied to the identification of compounds from reactions that yielded multiple products.

7.3.4 Multi-Product Reactions

The same LC-MS negative ion mode screening strategy described earlier was also performed on the reaction products of luteolin, eriodictyol, quercetin, hesperetin and myricetin. Two or three different glucuronides were identified in each elution profile based on the diagnostic loss of a glucuronic acid moiety (-176 Da) in the CID mass spectra. Once identified as glucuronides, these products were subjected to MS^3 analysis to yield the aglycon-specific fragmentation patterns shown in Figure 7.3. The monoglucuronidated flavonoid isomers in each reaction mixture yielded almost identical MS^3 profiles, though only the results from the earliest-eluting isomer is shown. All of these spectra matched the MS/MS spectra obtained from standards of the flavonoid aglycons.

After confirming that the reaction products were monoglucuronides and identifying the aglycon structures, CID of the $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ complexes was used to identify the glucuronidation positions in a manner similar to that described above. The MS/MS fragmentation patterns of the earliest-eluting isomer from each mixture are shown in Figure 7.4, and the characteristic

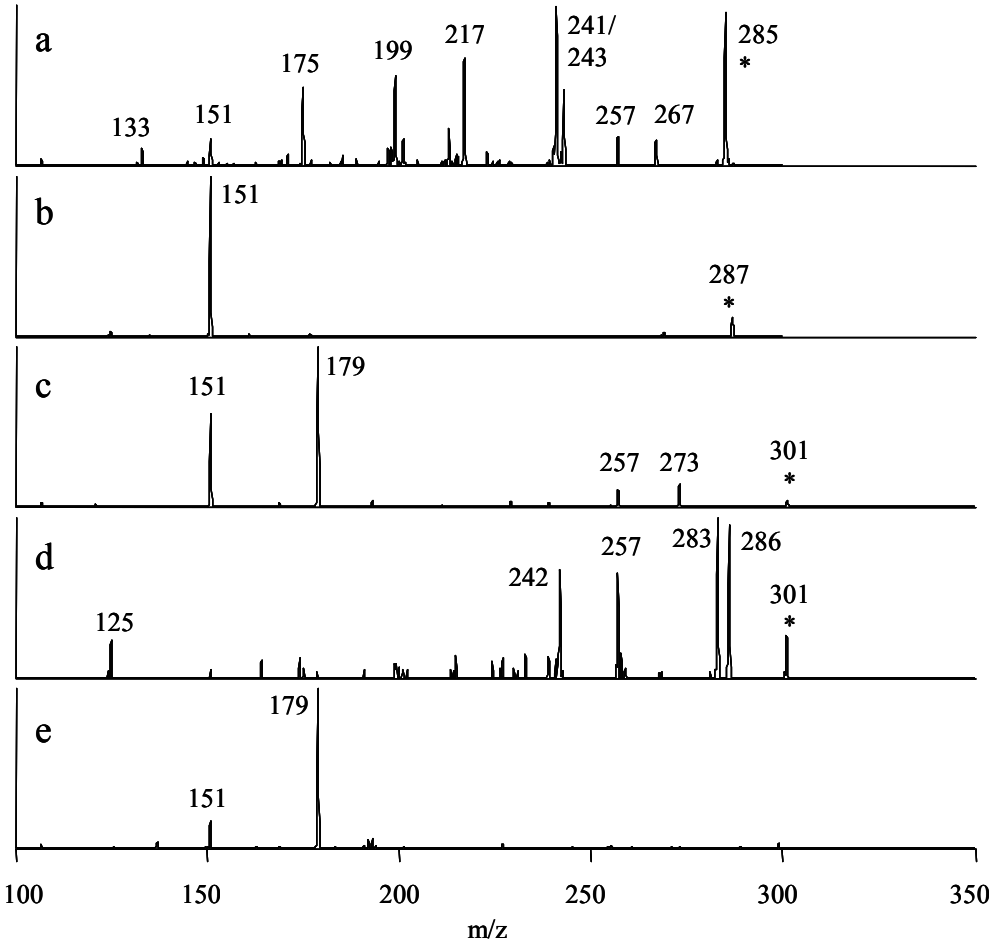


Figure 7.3. MS^n spectra used to confirm the identities of the aglycon skeletons of deprotonated flavonoid monoglucuronides (earliest eluting isomer from each mixture is shown). a) luteolin monoglucuronide, 461→285→ b) eriodictyol monoglucuronide, 463→287→ c) quercetin monoglucuronide, 477→301→ 268→ d) hesperetin monoglucuronide, 477→301→ e) myricetin monoglucuronide, 493→317→.

array of fragments indicate 7-O-glucuronidation in each case. The flavanone (eriodictyol and hesperetin) 7-O-glucuronide complexes again yield the highest abundance of the –Agl and several other fragment ions, while the flavone (luteolin) 7-O-glucuronide complex shows the lowest abundances of these

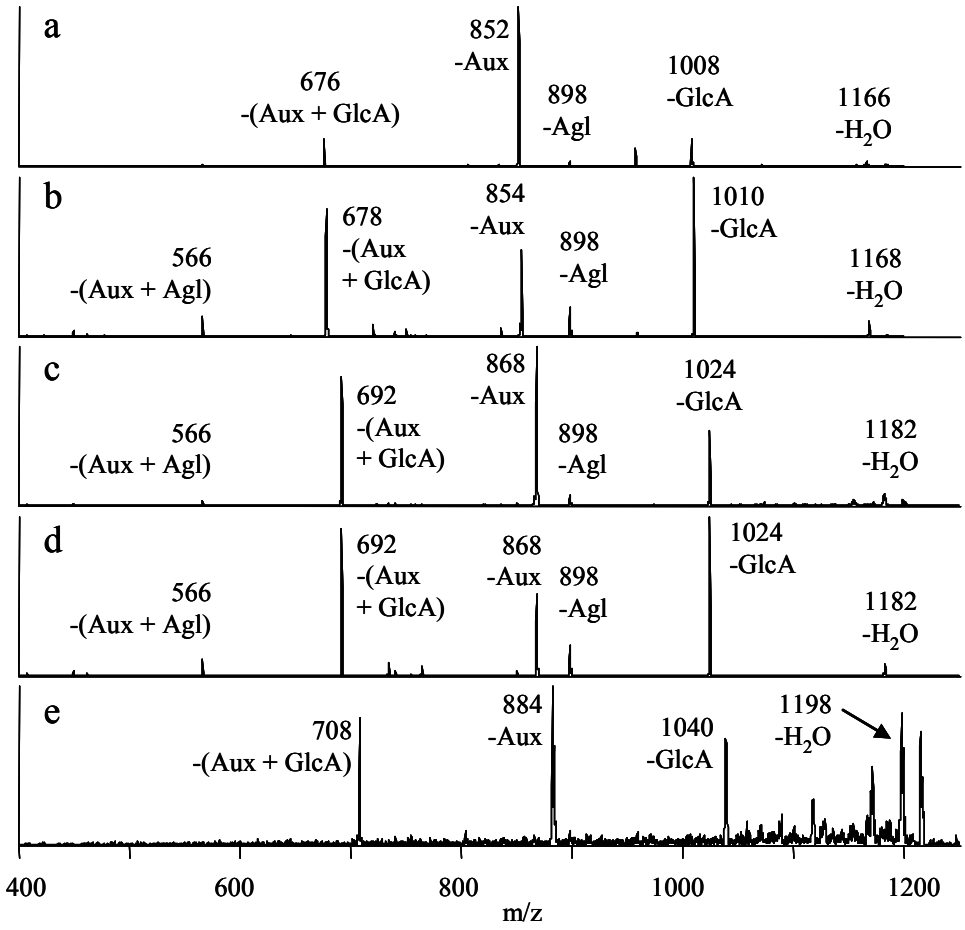


Figure 7.4. CID mass spectra of $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ complexes of earliest eluting flavonoid monoglucuronides from multi-product reactions. a) FG=luteolin, 1184→ b) FG=eriodictyol, 1186→ c) FG=quercetin, 1200→ d) FG=hesperetin, 1200→ e) FG=myricetin, 1216→.

fragment ions. The relative yield of 7-O-glucuronides was very low in the quercetin and myricetin reaction mixtures based on the intensities of these components in the chromatographic profiles, and 75 μL injections were required to obtain good mass spectra. The low signal intensity of myricetin 7-O-

glucuronide resulted in a very noisy MS/MS spectrum from the complex, especially in the high mass range. The diagnostic –Agl fragment ion is indistinguishable from the noise.

The enzymatic reactions involving eriodictyol and hesperetin yielded only one product in addition to the 7-O-glucuronide, while the remaining flavonoids produced two additional products. All of these products eluted after the respective 7-O-glucuronides. The CID mass spectra from the metal complexes of the latest-eluting isomers in each mixture are shown in Figure 7.5. These fragmentation patterns are indicative of B-ring-glucuronidation. As stated earlier, the two characteristic fragmentation pathways of complexes containing B-ring-glucuronides are –Aux and –(Aux + GlcA). The fragment ion abundances from these B-ring-glucuronide metal complexes appear to be partially dependent on the class of the flavonoid, as noted earlier for the 7-O-glucuronide complexes. While all of the spectra show both of the characteristic fragment ions of B-ring-glucuronides, the metal complex of the flavone (luteolin) B-ring-glucuronide strongly favors the loss of the auxiliary ligand, while the complexes of the flavanone (eriodictyol and hesperetin) B-ring-glucuronides preferentially yield the –(Aux + GlcA) ion. The flavonol (quercetin and myricetin) B-ring-glucuronide complexes display intermediate behavior, yielding similar amounts of each of these two fragment ions. Hence, knowledge of the flavonoid class helps in the interpretation of the fragmentation patterns of these metal complexes.

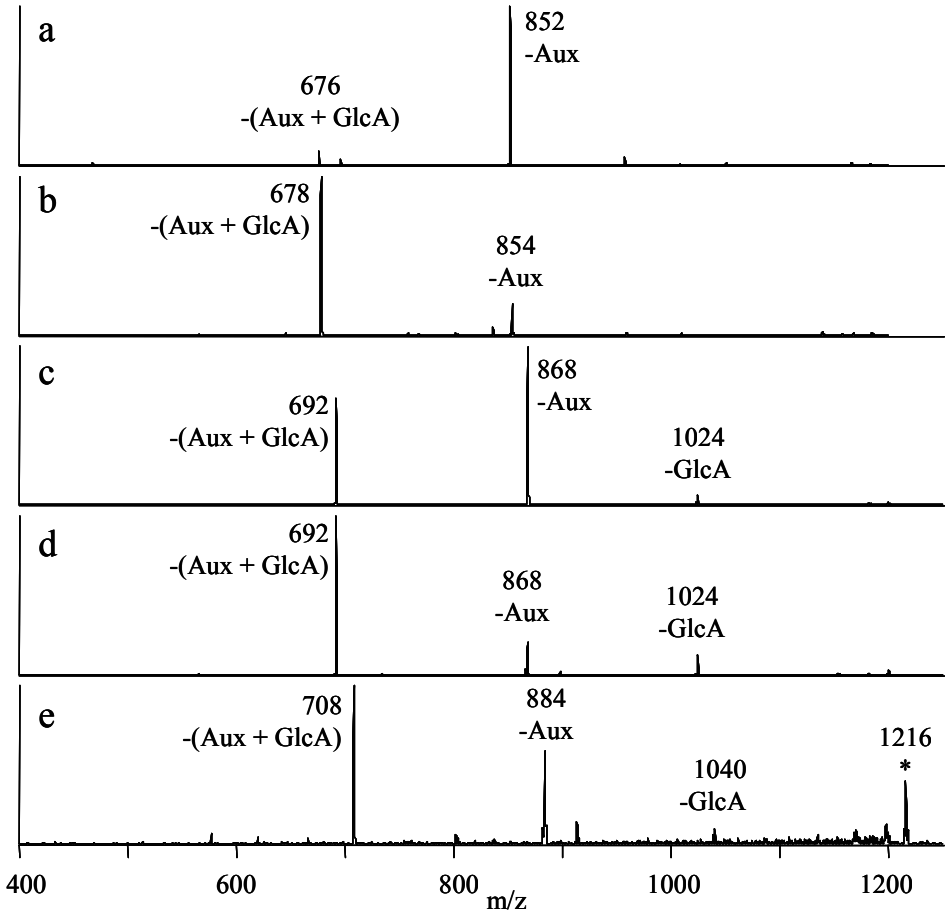


Figure 7.5. CID mass spectra of [Co(II) (FG-H) $(4,7\text{-dpphen})_2]^+$ complexes of latest eluting flavonoid monoglucuronides from multi-product reactions. a) FG=luteolin, 1184→ b) FG=eriodictyol, 1186→ c) FG=quercetin, 1200→ d) FG=hesperetin, 1200→ e) FG=myricetin, 1216→.

Some of the spectra also contain small amounts of the –GlcA fragment ion, which is usually associated with 7-O-glucuronidation. For the hesperetin glucuronide data in Figure 7.5 d, this is likely a contribution from the earlier-eluting hesperetin 7-O-glucuronide, as the two compounds were only partially

separated. However, the -GlcA fragment ion appears to be an intrinsic part of the spectrum for the complexes of the quercetin and myricetin B-ring-glucuronides because there are no co-eluting isomers in these cases. A reasonable guideline is that the MS/MS of flavone 7-O-glucuronide complexes displays at least 15% relative abundance of the –GlcA fragment ion (Figures 7.2 a, 7.2 c and 7.4 a), but this fragment ion is absent from the MS/MS of flavone B-ring-glucuronide complexes (Figure 7.5 a). For flavonols, the same product ion appears ~50% relative abundance for the 7-O-glucuronides (Figures 7.2 d, 7.4 c and 7.4 e), but at ~10% relative abundance for B-ring-glucuronides (Figures 7.5 c and 7.5 e). Since product ion distributions are dependent on collision energy,[31] these guidelines are only applicable to the conditions used in this set of experiments.

The collision energy used in the current set of experiments was specifically chosen to take advantage of a subtle difference in the fragmentation of the complexes of quercetin 4′-O-glucuronide and quercetin 3′-O-glucuronide.[31] When a collision voltage in the range of 1.5-1.8 V was used,[31] it was noted that a greater abundance of the –Aux fragment ion relative to the –(Aux + GlcA) fragment ion, as occurs in Figure 7.5 c, is indicative of quercetin 3′-O-glucuronide. If the relative abundances are reversed, this is characteristic of quercetin 4′-O-glucuronide, and this was observed for an isomeric compound in the quercetin reaction mixture that eluted just prior to quercetin 3′-O-glucuronide (data not shown). The retention time guidelines also support the identification of

these two products as quercetin 4′-O-glucuronide and quercetin 3′-O-glucuronide, in order of elution.

There are no known rules for differentiating the 3′-O- and 4′-O-glucuronides of flavonoids other than quercetin. Hence other means are required to precisely identify the remaining B-ring-glucuronides. The hesperetin B-ring-glucuronide is easiest to assign. Hesperetin has only one B ring hydroxyl group, so the unknown compound must be hesperetin 3′-O-glucuronide. Like quercetin, both the luteolin and myricetin reaction mixtures also display two glucuronide species that elute after the 7-O-glucuronide. As luteolin has two B ring hydroxyl groups, the two unknowns are assigned as luteolin 4′-O-glucuronide and luteolin 3′-O-glucuronide, in order of elution. These assignments were possible despite the failure of luteolin 4′-O-glucuronide to form significant amounts of the metal complex in question, for reasons that remain unknown. Both late-eluting myricetin glucuronides form complexes which dissociate to provide the same two major fragment ions shown in Figure 7.5 e. Myricetin has three B ring hydroxyl groups but two are equivalent, so the two isomers are identified as myricetin 4′-O-glucuronide and myricetin 3′-O-glucuronide, in order of elution. The eriodictyol derivative is the most difficult to assign because there are two B ring hydroxyl groups but only one B-ring-glucuronide is formed. While it is impossible to precisely identify this compound based on the available data, it is hypothesized to

be eriodictyol 3′-O-glucuronide, not eriodictyol 4′-O-glucuronide, for reasons that will be explained in the next section.

An estimate of the relative product yields of these multi-product reactions was made based on the peak areas of the initial negative ion mode MS scan (Table 7.2). Some of our results can be compared with data presented by Boersma et al.,[19] who reported product distributions for reactions of luteolin and quercetin with UGT1A1 based on HPLC, LC-MS and NMR spectroscopy. Their observed product distribution was 21:29:50 for the 7-O-, 3′-O- and 4′-O-glucuronides of luteolin, which is similar to our results. The distribution for quercetin was 6:85:9, which also parallels our data. As in the current study, quercetin 3-O-glucuronide was not observed as a reaction product.

Table 7.2. Estimated product distributions from multi-product reactions

aglycon	% 7-O-GlcA	% 3′-O-GlcA	% 4′-O-GlcA
eriodictyol	40	60*	–
hesperetin	70	30	–
luteolin	10	30	60
quercetin	trace	90	10
myricetin	trace	70	30

All values were rounded to the nearest 10%
* this compound may be eriodictyol 4′-O-glucuronide

7.3.5 Structure-Activity Relationships

The flavonoids that produce a single monoglucuronidated product and those which produce multiple products constitute two distinct groups,

distinguished by the presence or the absence of a 3′ hydroxyl group. The sole reaction product for compounds that lack this structural feature is the 7-O-glucuronide. The regioselectivity of UGT1A1 shifts toward the flavonoid B ring when a 3′ hydroxyl group is present, but the extent to which this happens depends on other structural features of the flavonoid. The two flavanones with a 3′ hydroxyl group, eriodictyol and hesperetin, form only one product in addition to the 7-O-glucuronide. The additional product of hesperetin was identified as the 3′-O-glucuronide, but the additional product of eriodictyol could not be conclusively determined. However, the important activating properties of the 3′ hydroxy group suggest that eriodictyol 3′-O-glucuronide is the product that is formed. For both eriodictyol and hesperetin, there remains a significant yield of the 7-O-glucuronide (relative yields of 40% and 70%, respectively). Luteolin is the only flavone in the study that features a 3′ hydroxyl group. There is a larger shift toward B-ring-glucuronidation as luteolin 7-O-glucuronide makes up only about 10% of the product. For the flavonols containing the 3′ hydroxyl group (quercetin and myricetin), only trace amounts of their 7-O-glucuronides were formed. Hence, while the presence or absence of the 3′ hydroxyl group is the major determinant of the regioselectivity of glucuronidation, there are secondary effects due to flavonoid class.

The structural features that govern the regioselectivity of the glucuronidation reaction (the presence or absence of the 3′ hydroxyl group, the

C2-C3 double bond, and the 3 hydroxyl group) are also important factors in the antioxidant capability of flavonoids, and may act synergistically in that capacity as well.[60] The same structural features have also been cited as important determinants of the antiproliferative effects of flavonoids against several cancer cell lines.[61] It is interesting to note that while the presence of the ortho-catechol group (i.e. the 3′ and 4′ hydroxyl groups) is often claimed to be a source of beneficial flavonoid activity, our work shows a tendency for this group to be preferentially metabolized by UGT1A1, which would potentially mitigate the bioactivity of these molecules. On the other hand, the B-ring-glucuronides of quercetin retain the inhibitory effects of the aglycon against xanthine oxidase and lipoxygenase better than when quercetin is conjugated at other positions.[17]

7.4 CONCLUSIONS

A three-part tandem mass spectrometry strategy that entails MS/MS analysis of deprotonated metabolites to pinpoint those that are glucuronides, then MS^3 analysis to elucidate the specific aglycon structure, then MS/MS analysis of metal complexes of the type $[Co(II)\ (FG\text{-}H)\ (4,7\text{-dpphen})_2]^+$ allows confident identification of enzymatically-synthesized flavonoid glucuronides, including the conjugation site. This strategy was used to determine the regioselectivity of UGT1A1 toward flavonol, flavone and flavanone substrates. The 7 position of the flavonoid is the sole site of glucuronidation except when a 3′ hydroxyl group

is present. A 3′ hydroxyl group promotes the formation of B-ring-glucuronides in addition to 7-O-glucuronides. The flavonoid class determines the product distribution in such cases. This result represents a significant advance in understanding the of flavonoid metabolism. Studies involving other UGT isozymes are planned. Furthermore, we have demonstrated the success of metal complexation methods in the identification of new flavonoid monoglucuruonides with the assistance of HPLC retention time analysis. Neither NMR nor authenticated standards were required to make the assignments. Of the eighteen flavonoid monoglucuronides produced, only one remains ambiguous (the eriodictyol B-ring-glucuronide). Insights regarding the effect of flavonoid class on the fragmentation of the metal complexes will prove useful for future applications of this method.

7.5 REFERENCES

(1) Manach C, Donovan JL. *Free Radical Research* **2004**, *38*, 771-785.
(2) Williamson G, Day AJ, Plumb GW, Couteau D. *Biochemical Society Transactions* **2000**, *28*, 16-22.
(3) Prasain JK, Wang CC, Barnes S. *Free Radical Biology and Medicine* **2004**, *37*, 1324-1350.
(4) Hong YJ, Mitchell AE. *Chemical Research in Toxicology* **2006**, *19*, 1525-1532.
(5) Williamson G, Barron D, Shimoi K, Terao J. *Free Radical Research* **2005**, *39*, 457-469.
(6) Wittig J, Herderich M, Graefe EU, Veit M. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2001**, *753*, 237-243.
(7) Day AJ, Williamson G. *British Journal of Nutrition* **2001**, *86*, S105-S110.

(8) Sesink ALA, O'Leary KA, Hollman PCH. *Journal of Nutrition* **2001**, *131*, 1938-1941.
(9) Paganga G, Rice-Evans CA. *Febs Letters* **1997**, *401*, 78-82.
(10) Aziz AA, Edwards CA, Lean MEJ, Crozier A. *Free Radical Research* **1998**, *29*, 257-269.
(11) Boyle SP, Dobson VL, Duthie SJ, Kyle JAM, Collins AR. *European Journal of Nutrition* **2000**, *39*, 213-223.
(12) Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G. *Free Radical Research* **2001**, *35*, 941-952.
(13) Mullen W, Edwards CA, Crozier A. *British Journal of Nutrition* **2006**, *96*, 107-116.
(14) Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C. *European Journal of Clinical Nutrition* **2003**, *57*, 235-242.
(15) Mullen W, Graf BA, Caldwell ST, Hartley RC, Duthie GG, Edwards CA, Lean MEJ, Crozier A. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 6902-6909.
(16) Walle T, Walle UK, Halushka PV. *Journal of Nutrition* **2001**, *131*, 2648-2652.
(17) Day AJ, Bao YP, Morgan MRA, Williamson G. *Free Radical Biology and Medicine* **2000**, *29*, 1234-1243.
(18) Abe K, Katayama H, Suzuki A, Yumioka E. *Shoyakugaku Zasshi* **1993**, *47*, 402.
(19) Boersma MG, van der Woude H, Bogaards J, Boeren S, Vervoort J, Cnubben NHP, van Iersel MLPS, van Bladeren PJ, Rietjens IMCM. *Chemical Research in Toxicology* **2002**, *15*, 662-670.
(20) Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, **1970**.
(21) Matsumoto H, Ikoma Y, Sugiura M, Yano M, Hasegawa Y. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 6653-6659.
(22) Needs PW, Kroon PA. *Tetrahedron* **2006**, *62*, 6862-6868.
(23) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(24) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(25) Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
(26) Davis BD, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1883-1890.
(27) Zhang JM, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1761-1770.
(28) Zhang JM, Wang JM, Brodbelt JS. *Journal of Mass Spectrometry* **2005**, *40*, 350-363.

(29) Pikulski M, Aguilar A, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2007**, *18*, 422-431.
(30) Wojcinska M, Williams J, Mabry TJ, Ahmed AA, Davis BD, Toth G, El-Sayed NH, Matlawska I, Clevinger J. *Natural Product Communications* **2006**, *1*, 941-948.
(31) Davis BD, Needs PW, Kroon PA, Brodbelt JS. *Journal of Mass Spectrometry* **2006**, *41*, 911-920.
(32) Ebner T, Burchell B. *Drug Metabolism and Disposition* **1993**, *21*, 50-55.
(33) Green MD, Oturu EM, Tephly TR. *Drug Metabolism and Disposition* **1994**, *22*, 799-805.
(34) King CD, Green MD, Rios GR, Coffman BL, Owens IS, Bishop WP, Tephly TR. *Archives of Biochemistry and Biophysics* **1996**, *332*, 92-100.
(35) Green MD, Tephly TR. *Drug Metabolism and Disposition* **1998**, *26*, 860-867.
(36) Cheng ZQ, Radominska-Pandya A, Tephly TR. *Drug Metabolism and Disposition* **1999**, *27*, 1165-1170.
(37) King CD, Rios GR, Green MD, Tephly TR. *Current Drug Metabolism* **2000**, *1*, 143-161.
(38) Vaidyanathan JB, Walle T. *Drug Metabolism and Disposition* **2002**, *30*, 897-903.
(39) Zhang L, Lin G, Zuo Z. *Life Sciences* **2006**, *78*, 2772-2780.
(40) Plumb GW, O'Leary K, Day AJ, Williamson G. In *Methods in Polyphenol Analysis*; Santos-Buelga C, Williamson G, Ed.; The Royal Society of Chemistry: Cambridge, **2003**.
(41) McClellan JE, Murphy JP, Mulholland JJ, Yost RA. *Analytical Chemistry* **2002**, *74*, 402-412.
(42) Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.
(43) Davis BD, Kroon PA, Brodbelt JS. *In Vivo* Human Metabolites of Flavonols and Flavanones. *55th ASMS Conference on Mass Spectrometry and Allied Topics*, Indianapolis, **2007**.
(44) Ma YL, Li QM, VandenHeuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(45) Justesen U. *Journal of Mass Spectrometry* **2001**, *36*, 169-178.
(46) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
(47) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
(48) Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C. *Journal of Neurochemistry* **2003**, *85*, 180-192.
(49) Proteggente AR, Basu-Modak S, Kuhnle G, Gordon MJ, Youdim K, Tyrrell R, Rice-Evans CA. *Photochemistry and Photobiology* **2003**, *78*, 256-261.

(50) Robards K, Antolovich M. *Analyst* **1997**, *122*, 11R-34R.
(51) Schieber A, Keller P, Streker P, Klaiber I, Carle R. *Phytochemical Analysis* **2002**, *13*, 87-94.
(52) de Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2003**, *984*, 45-58.
(53) Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Viladomat F, Bastida J, Codina C. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 553-563.
(54) Dugo P, Lo Presti M, Ohman M, Fazio A, Dugo G, Mondello L. *Journal of Separation Science* **2005**, *28*, 1149-1156.
(55) Lin LZ, Harnly JM. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 1084-1096.
(56) de Pascual-Teresa S, Rivas-Gonzalo JC. In *Methods in Polyphenol Analysis*; Santos-Buelga C, Williamson G, Ed.; The Royal Society of Chemistry: Cambridge, UK, **2003**.
(57) Mullen W, Boitier A, Stewart AJ, Crozier A. *Journal of Chromatography A* **2004**, *1058*, 163-168.
(58) Grayer RJ, Kite GC, Veitch NC, Eckert MR, Marin PD, Senanayake P, Paton AJ. *Biochemical Systematics and Ecology* **2002**, *30*, 327-342.
(59) Harborne JB, Boardley M. *Journal of Chromatography* **1984**, *299*, 377-385.
(60) Rice-Evans CA, Miller NJ, Paganga G. *Free Radical Biology and Medicine* **1996**, *20*, 933-956.
(61) Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. *Bioscience Biotechnology and Biochemistry* **1999**, *63*, 896-899.

Chapter 8: Homolytic and Heterolytic Saccharide Loss During Collision-Induced Dissociation of Flavonol 3-O-Glycosides

8.1 Introduction

Throughout the earlier chapters of this dissertation, it was demonstrated that identifying the aglycon portion of flavonoid conjugates is a fairly simple process. This is usually true if a mass spectrometer that provides multiple stages of collision-induced dissociation (CID), such as a quadrupole ion trap, is used. The first stage of fragmentation of deprotonated O-glycosyl flavonoids is typically the neutral loss of the saccharide moiety. After the saccharide moiety is lost, the second stage of fragmentation usually provides a wide variety of product ions that is highly specific to the flavonoid aglycon. In fact, these fragment ions usually closely match those yielded by deprotonated flavonoid aglycons standards. Identifying the structure of the aglycon portion of a flavonoid glycoside, therefore, is usually as simple as matching spectra between the unknown compound and a native flavonoid aglycon.[1-7]

However, in the course of identifying the flavonoid glycosides in a *Siphium albiflorum* extract (described in Section 5.3.4), a prominent exception to these generalizations was noted. The second stage of fragmentation of some of the unknown monoglycosyl flavonoids in the extract did not match any known aglycon fragmentation patterns. After a series of further tests, the aglycon portion of the unknown compounds was identified as a common flavonol, kaempferol,

despite the fact that the fragmentation pathways did not closely match those of kaempferol aglycon. As the overall goal of this body of research is to provide a means to confidently identify flavonoid conjugates using tandem mass spectrometry, this chapter will document this unusual phenomenon and attempt to explain why some flavonoid conjugates undergo atypical fragmentation behavior. A firm understanding of this behavior will make it less likely that incorrect conclusions will be drawn from the fragmentation patterns of flavonoid derivatives.

The investigation quickly centered on the phenomenon of homolytic saccharide cleavage, first described by Hvattum and Ekeberg.[8] It was shown that during the first stage of CID many deprotonated flavonoid glycosides undergo homolytic saccharide losses in addition to the usual heterolytic cleavages. Hvattum and Ekeberg found correlations between the ratio of homolytic vs. heterolytic cleavage products and the structures of the molecules, particularly the position of the saccharide group and the degree of hydroxylation on the B ring of the aglycon. Most relevant to this discussion is the positive correlation they found between the number of hydroxyl groups on the B ring of 3-O-glycosyl flavonols and the amount of homolytic saccharide cleavage. The unusual behavior of the kaempferol glycosides provides an exception to these general guidelines that is related to the complications in structural analysis. Understanding homolytic saccharide cleavage is of particular importance because researchers have

increasingly begun using this feature as an indicator of flavonoid structure.[9-14] Here an expanded study of the phenomenon of homolytic saccharide cleavage is presented, with a focus on the implications for flavonoid identification.

8.2 EXPERIMENTAL

Kaempferol 3-O-rutinoside, quercetin 3-O-glucoside, quercetin 3-O-galactoside and myricetin 3-O-rhamnoside were purchased from Extrasynthèse (Genay, France). Kaempferol 3-O-glucoside and quercetin 3-O-rhamnoside were purchased from Indofine (Somerville, NJ). Quercetin 3-O-arabinofuranoside and quercetin 3-O-xyloside were purchased from Apin Chemicals (Abingdon, UK). Quercetin 3-O-rutinoside was purchased from Sigma-Aldrich (St. Louis, MO). The extract from *Silphium albiflorum* was provided by Jeffrey Williams and Malgorzata Wojcinska of the University of Texas Department of Biology; a detailed extraction procedure is provided elsewhere.[15]

8.3 RESULTS AND DISCUSSION

The fragmentation pathways of deprotonated flavonoid O-glycosides are usually very simple. In general, the glycosidic bond is cleaved heterolytically causing the neutral loss of the saccharide and leaving behind the deprotonated aglycon portion of the molecule.[10,16-19] Sometimes a few other product ions are

also observed. In the case of molecules containing more than one saccharide, it varies as to whether the loss of individual monosaccharides occurs or whether the concurrent loss of all saccharide units predominates. Often both results are observed in the same MS/MS spectrum in varying proportions.[12,20]

Hvattum and Ekeberg described a variation on this pattern, in which saccharide moieties may be lost through homolytic cleavage in addition to typical heterolytic saccharide cleavage, resulting in radical product ions.[8] For the flavonol 3-O-glycosides, a positive correlation was reported between the number of hydroxyl groups on the B ring of the flavonoid and the proportion of saccharide losses in the form of radical species. In the current study, this trend was duplicated using the same set of analytes (Table 8.1). The collision energy in a QIT mass spectrometer is lower than in a triple-quad,[21] so the proportions of radical cleavage were lower than previously reported when typical QIT collision energies were employed. However, the amount of radical cleavage increases with

Table 8.1. Radical saccharide losses from deprotonated flavonol 3-O-glycosides

flavonoid glycoside	% radical loss (CID=25-30%)	% radical loss (CID=100%)	critical CID energy, Y_0-$H^{\bullet -}$	critical CID energy, Y_0^-
kaempferol 3-O-rutinoside*	5	12	28	33
quercetin 3-O-arabinofuranoside	9	31	29	28
quercetin 3-O-rhamnoside*	28	46	29	28
quercetin 3-O-xyloside	24	47	29	29
quercetin 3-O-glucoside*	24	53	30	29
quercetin 3-O-rutinoside*	31	55	30	29
quercetin 3-O-galactoside*	34	59	29	28
myricetin 3-O-rhamnoside*	76	85	30	29
kaempferol 3-O-glucoside	71	95	25	41

* indicates compounds studied by Hvattum and Ekeberg[8]

collision energy (as reported), and using a much higher energy yielded values similar to the previous study. The lack of homolytic cleavage from 4′-O-glycosides was also confirmed. It was difficult to produce enough of the radical species from the 7-O-glycosides to confirm the assertion that homolytic cleavage of these compounds is favored by fewer hydroxyl groups on the B ring. By this criterion, apigenin 7-O-glucoside should have produced the most homolytic cleavage products, and indeed it was one of the few 7-O-glycosides for which radical fragment ions were observed. As the amount observed was very small, it makes sense that no radical cleavage was observed from flavonoid 7-O-glycosides with a higher degree of B ring hydroxylation.

Although the results were confirmed for the same analytes used in the earlier study, an expanded sample set revealed that the correlation between B ring hydroxylation and radical saccharide cleavage of flavonol 3-O-glycosides is not perfect. This is due to the kaempferol 3-O-glycosides, which vary widely in their radical losses despite sharing the same parent aglycon. Kaempferol 3-O-rutinoside exhibited the lowest amount of radical cleavage of all the 3-O-glycosides, while kaempferol 3-O-glucoside exhibited the highest amounts. Kaempferol 3-O-galactoside, present in the *Silphium albiflorum* extract, behaved similarly to kaempferol 3-O-galactoside, but is omitted from Table 8.1 because this compound could not be analyzed from the extract under the same conditions as the purified standards. The variable behavior of the kaempferol glycosides

demonstrates that another factor besides B-ring hydroxylation is driving the homolytic cleavage of the saccharide moiety. In contrast, the 3-O-rutinoside, 3-O-glucoside, and 3-O-galactoside derviatives of quercetin exhibit nearly identical behavior in terms of homolytic vs. heterolytic cleavage; so the saccharide moiety by itself cannot be the reason for the disparity of the kaempferol derivatives. However, even the fragmentation of the quercetin 3-O-glycosides exhibits a small dependence on the identity of the saccharide. For example, quercetin 3-O-arabinofuranoside exhibits only half the radical saccharide cleavage as quercetin 3-O-galactoside. No simple general correlation between homolytic cleavage behavior and either the identity of the aglycon or the saccharide is apparent from the data. The structural features of the aglycon portion and the saccharide portion of the molecule interact in unpredictable ways.

Dissociation of the radical aglycon product ions $[Y_0\text{-H}]^{-\bullet}$ also yields unexpected results. Performing an additional stage of CID on the Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions of the flavonoid 3-O-glycosides proves that the two species dissociate via vastly different routes. Quercetin 3-O-glucoside provides a good example of this (Figure 8.1). The Y_0^- ion (m/z 301) generates the same product ions as deprotonated quercetin ($[M\text{-H}]^-$, m/z 301). The main product ions in each case are the $^{1,3}A^-$ ion of m/z 151, the $^{1,2}A^-$ ion of m/z 179, and ions stemming from the loss of CO_2 and CO of m/z 257 and m/z 273, respectively.[1,2] In contrast, the $[Y_0\text{-H}]^{-\bullet}$ radical product ion of qurecetin 3-O-glucoside (m/z 300) produces just two

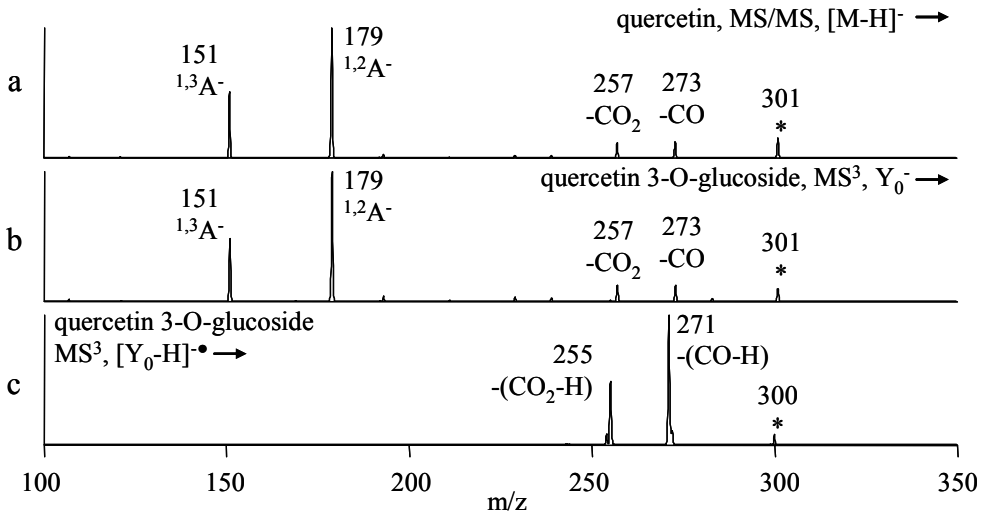


Figure 8.1. CID spectra quercetin and quercetin 3-O-glucoside. (a) MS/MS of deprotonated quercetin. (b) Second-generation ions produced by Y_0^- ion of quercetin 3-O-glucoside. (c) Second-generation ions produced by $[Y_0\text{-H}]^{-\bullet}$ ion of quercetin 3-O-glucoside.

second-generation product ions of m/z 255 and m/z 271, which do not appear in the CID mass spectrum of the nonradical Y_0^- anion. The structures of these two new product ions are hypothesized to be similar to m/z 257 and m/z 273, but involving the loss of additional hydrogen atoms. Plausible structures of these ions are proposed (Figure 8.2). Similar structures have also been proposed in other contexts.[1,22]

If the Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ first-generation product ions of quercetin 3-O-glucoside are dissociated together by using an isolation window that encompasses both, the mixture of second-generation product ions that is obtained is dependent mainly on the collision energy of the first stage of fragmentation. Figure 8.3

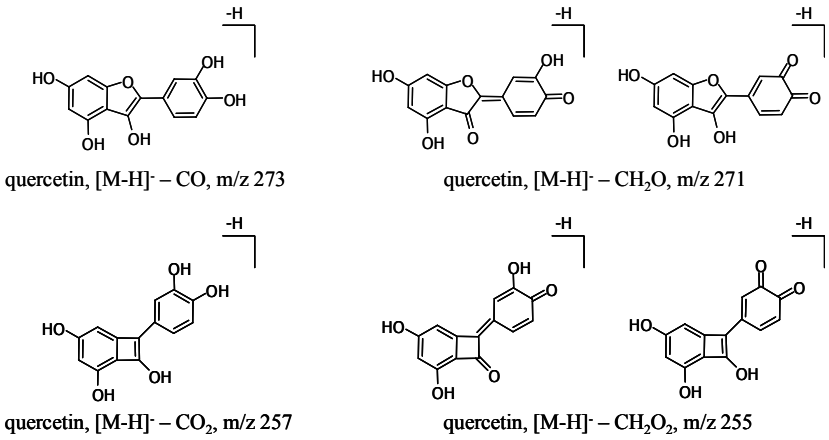


Figure 8.2. Proposed structures of ions yielded by quercetin glycosides.

shows the results of several experiments performed with varying collision energies. First the collision energy of the initial stage of CID was varied while the second stage of CID was held constant at 27%, which is low enough to preserve the some of the first-generation Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions in the MS^3 spectrum. When the first stage of CID is also performed at 27% collision energy, the Y_0^- ion of m/z 301 is more abundant than the $[Y_0\text{-H}]^{-\bullet}$ ion of m/z 300 (Figure 8.3 a). Increasing the fragmentation energy of the first CID step to 100% boosts the proportion of homolytic saccharide cleavage such that the $[Y_0\text{-H}]^{-\bullet}$ ion is more abundant (Figure 8.3 b). These differences in turn affect the abundances of the second-generation product ions. The product ions of m/z 255 and m/z 271, which are exclusively produced from the first-generation $[Y_0\text{-H}]^{-\bullet}$ ion, become more abundant compared to the other second-generation product ions as the proportion

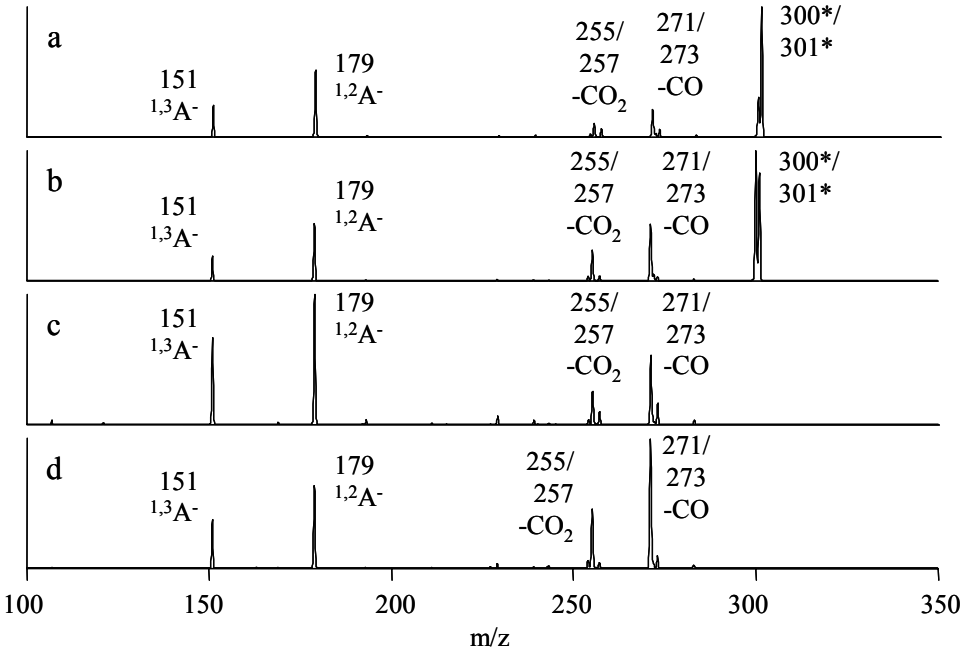


Figure 8.3. MS^3 spectra of deprotonated quercetin 3-O-glucoside with varying CID energy. The Y_0^- ion and the $[Y_0\text{-H}]^{-\bullet}$ ion were dissociated together in the second stage of fragmentation. The fragmentation energies of the two CID stages are: (a) 27%, 27% (b) 100%, 27% (c) 27%, 50% (d) 100%, 50%.

of the $[Y_0\text{-H}]^{-\bullet}$ ion increases due to higher CID energy in the first step. The same trend is apparent when 50% CID energy is used in the second fragmentation stage instead of 27% (Figure 8.3, c and d). In this case, although the Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions do not survive the second CID stage, the increase in the abundances of m/z 255 and m/z 271 is clearly due to higher levels of $[Y_0\text{-H}]^{-\bullet}$ produced in the first CID step. On the other hand, the second-stage CID energy has less of an effect on the relative abundance of second-generation product ions, demonstrated by keeping the second stage CID energy constant while varying the CID energy in the first stage (compare Figure 8.3 a and c or b and d).

In all of these spectra, the product ions (m/z 255 and m/z 271) from the first-generation $[Y_0\text{-H}]^{-\bullet}$ ion are more abundant than the corresponding product ions (m/z 257 and m/z 273) from the first-generation Y_0^- ion. This trend is observed among the other quercetin 3-O-glycosides with subtle differences based on the saccharide (Figure 8.4). The most noticeable difference occurs with quercetin 3-O-arabinofuranoside, which shows almost no preference for the first-generation $[Y_0\text{-H}]^{-\bullet}$ ions product ions over the corresponding Y_0^- product ions (Figure 8.4 f). Not surprisingly, this compound also shows a significantly lower tendency towards homolytic saccharide cleavage than the other quercetin-3-O-glycosides (Table 8.1). Additionally, this compound experiences relatively less of the fragmentation pathways that involve the loss of CO and CO_2, either as radical losses or normal losses. These significant differences are surprising because the spectra are examples of MS^3 scans taken following the loss of the saccharide moiety. Since the parent compounds differ only by the identity of the saccharide, it might be expected that the results of the second CID step would be the same in all cases. Because this does not happen, it must be concluded that even though the only distinguishing feature of these compounds is lost in the first fragmentation stage, the resultant first-generation product ions are dissimilar enough structurally to produce different distributions of second-generation product ions.

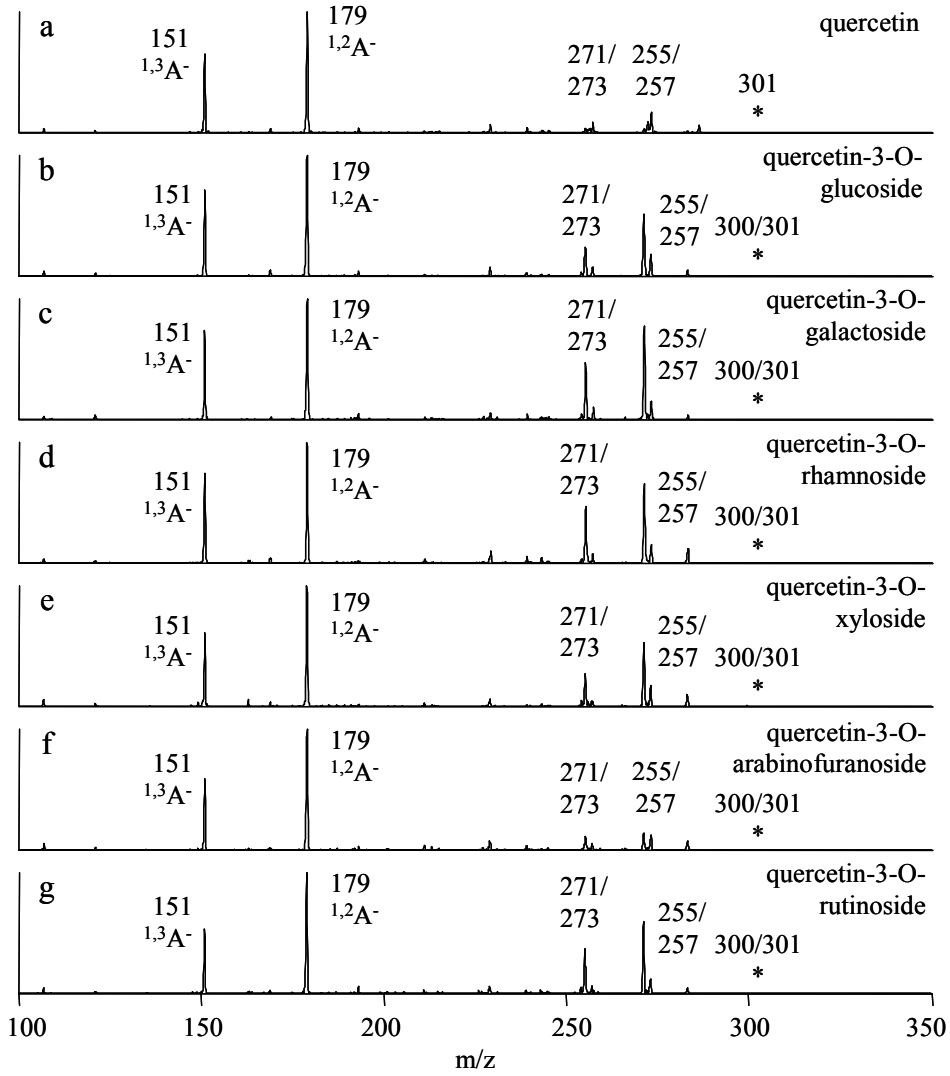


Figure 8.4. CID spectra of quercetin and quercetin 3-O-glycosides. The CID energy is 50% in all cases. (a) MS/MS of deprotonated quercetin, precursor ion $[M-H]^-$ (b-g) MS^3 of quercetin 3-O-glycosides, precursor ions Y_0^- and $[Y_0-H]^{-\bullet}$.

Many of the same trends observed for the quercetin 3-O-glycosides are also seen for the kaempferol 3-O-glycosides and the myricetin 3-O-glycosides. In the case of the quercetin 3-O-glycosides, the second-generation CID products that

are descended from the first-generation $[Y_0\text{-H}]^{-\bullet}$ ion do not greatly interfere with the identification of the quercetin aglycon. The $^{1,3}A^-$ and $^{1,2}A^-$ product ions still appear in the MS^3 spectra of the quercetin-3-O-glycosides, and the unexpected fragment ions m/z 255 and m/z 271 are clearly associated with m/z 257 and m/z 273, which are also still observed. However, the myricetin and kaempferol 3-O-glycosides in this study are more likely to cause errors in identification due to the dissimilarities between the first-generation product ions of the aglycon and the second-generation product ions of the glycosides.

Myricetin 3-O-rhamnoside produces both Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions upon CID, and the two species again produce different second-generation product ions. Those produced by the Y_0^- ion (Figure 8.5 b) are identical to those produced by the $[M\text{-H}]^-$ ion of the native deprotonated aglycon (Figure 8.5 a). The $[Y_0\text{-H}]^{-\bullet}$ ion produces a completely new series of product ions (Figure 8.5 c), somewhat analogous to those produced by $[Y_0\text{-H}]^{-\bullet}$ species of the quercetin 3-O-glycosides. But when dissociated together, the second generation product ions from the $[Y_0\text{-H}]^{-\bullet}$ species dominate the spectrum (Figure 8.5 d), giving a completely different signature than deprotonated myricetin. This is particularly true because deprotonated myricetin does not produce any significant product ions above m/z 200. If MS^3 analysis is used to identify the aglycon portion of myricetin 3-O-rhamnoside, proper identification will be hindered if the homolytic cleavage

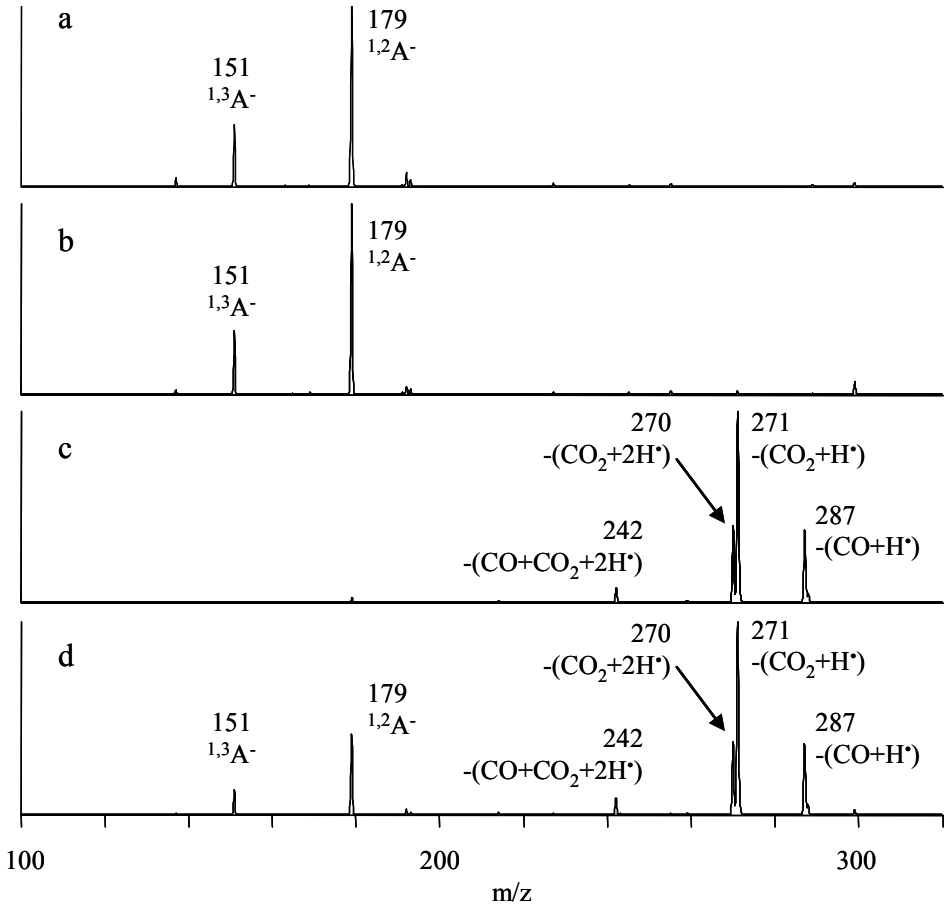


Figure 8.5. Negative ion mode CID spectra of myricetin and myricetin 3-O-rhamnoside. (a) MS/MS of deprotonated myricetin, $[M-H]^-$ (m/z 317), CID=50%; (b) MS^3 of myricetin 3-O-rhamnoside, precursor ion Y_0^- (m/z 317), CID=27%, 50%; (c) MS^3 of myricetin 3-O-rhamnoside, precursor ion $[Y_0-H]^{-\bullet}$ (m/z 316), CID=27%, 50%; (d) MS^3 of myricetin 3-O-rhamnoside, precursor ions Y_0^- and $[Y_0-H]^{-\bullet}$, CID=27%, 50%.

product $[Y_0-H]^{-\bullet}$ is dissociated along with or instead of the typical heterolytic cleavage product, Y_0^-.

The possibility of misidentification is even greater in the case of the kaempferol 3-O-glycosides. The negative ion CID spectrum of kaempferol is

very complicated, with nearly a dozen major fragment ions and many more minor ones (Figure 8.6 a). The Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions of kaempferol 3-O-glucoside produce different second-generation product ions as expected, but neither shows nearly the same complexity in their fragmentation pathways as native kaempferol. Whether dissociated separately or together, it is impossible to match the MS^3 spectrum of kaempferol 3-O-glucoside with the MS/MS spectrum of kaempferol. Thus the aglycon portion of this molecule cannot be determined by simple comparison with kaempferol. All three kaempferol 3-O-glycosides (including kaempferol 3-O-galactoside) behave similarly when the Y_0^- and $[Y_0\text{-H}]^{-\bullet}$ ions are dissociated separately, but a difference is observed when these two species are dissociated together due to the relative stabilities of these two species (Table 8.1). Not only does kaempferol 3-O-glucoside yield more of the $[Y_0\text{-H}]^{-\bullet}$ product ion, this ion requires less CID energy to fragment than the Y_0^- product ion. As a result, the second-generation product ions from the $[Y_0\text{-H}]^{-\bullet}$ ion dominate the MS^3 spectrum (not shown). In contrast, kaempferol 3-O-rutinoside produces much less of the $[Y_0\text{-H}]^{-\bullet}$ product ion, and its dissociation energy is similar to that of the non-radical product ion Y_0^-. Thus the MS^3 spectrum favors the second-generation product ions from the Y_0^- ion (Figure 8.6 d). But this spectrum is still quite different from the MS/MS spectrum of deprotonated kaempferol (Figure 8.6 a).

Hence it is not always so simple to use tandem mass spectrometry to identify the aglycon portion of flavonoid conjugates. Among the compounds

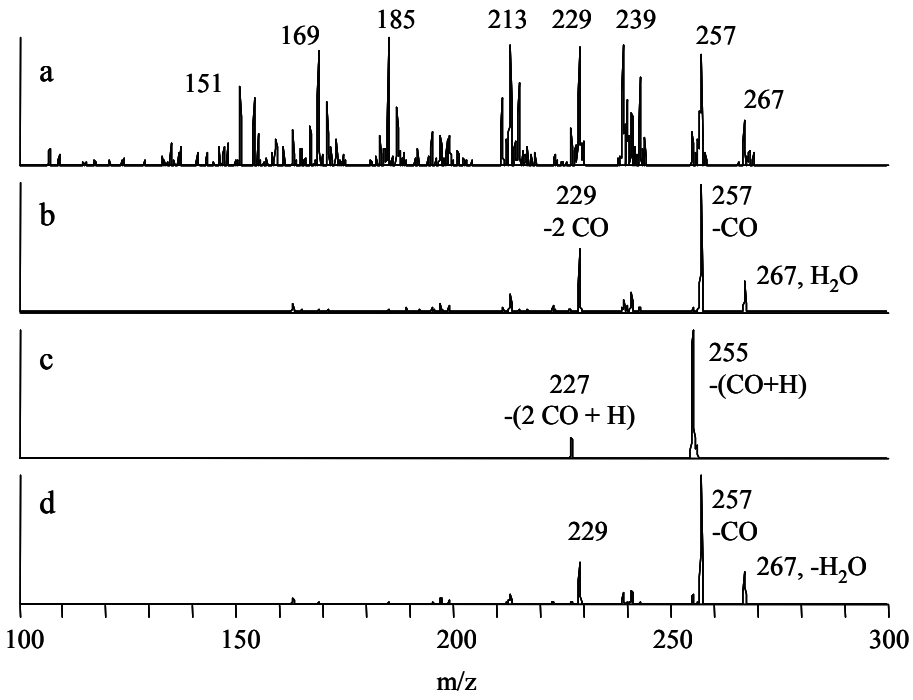


Figure 8.6. Negative ion mode CID spectra of kaempferol and kaempferol 3-O-glycosides. (a) MS/MS of deprotonated kaempferol, $[M-H]^-$ (m/z 285), CID=50%; (b) MS^3 of kaempferol 3-O-glucoside, precursor ion Y_0^- (m/z 285), CID=31%, 50%; (c) MS^3 of kaempferol 3-O-glucoside, precursor ion $[Y_0-H]^{-\bullet}$ (m/z 284), CID=31%, 50%; (d) MS^3 of kaempferol 3-O-rutinoside, precursor ions Y_0^- and $[Y_0-H]^{-\bullet}$, CID=27%, 50%.

available for study, the MS^3 spectra of the kaempferol 3-O-glycosides and of myricetin 3-O-rhamnoside showed the greatest deviance from the MS/MS spectra of their parent aglycons. The kaempferol 3-O-glycosides in the *Silphium albiflorum* extract (described in Section 5.3.4) were therefore confirmed as kaempferol derivatives by comparison to a standard of kaempferol 3-O-glucoside, not native kaempferol. On the other hand, the MS^3 spectrum of kaempferol 7-O-glucuronide showed a fragmentation pattern similar to that of kaempferol aglycon

(see Figure 7.1 d). Kaempferol 3-O-glucuronide, identified from a cell culture sample (Section 6.3.5), also provided a rich and varied fragmentation pattern similar to native kaempferol. These examples further illustrate the convoluted interactions between saccharide and glycosylation site in influencing the characteristics of the MS^3 spectra.

8.4 CONCLUSIONS

The MS^3 spectra of some flavonoid derivatives differ significantly from the MS/MS spectra of their parent aglycons. This phenomenon appears to be related to radical saccharide cleavage, which is in turn influenced by the glycosylation position, the nature of the conjugated saccharide and the structure of the aglycon portion of the molecule. The explanation for why different compounds undergo more or less radical saccharide cleavage is still unknown, but molecular modeling is ongoing to help answer this question. The practical implication of the observations presented in this chapter is that the aglycon portion of flavonoid derivatives that exhibit this behavior cannot be easily identified by performing sequential fragmentation and comparing the results to a library of MS/MS spectra obtained from native aglycons, a strategy that was heavily utilized in the applications described in previous chapters. The compounds most likely to cause problems are the kaempferol 3-O-glycosides and the myricetin 3-O-glycosides. To identify the aglycon portions of these

molecules, it may be necessary to compare the MS^3 fragmentation to that of another 3-O-glycoside of that shares the same parent aglycon. Vigilance is needed to avoid mistaken assignments, but as long as the exceptions are known, even these compounds may be identified with confidence.

8.5 REFERENCES

(1) Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
(2) Hughes RJ, Croley TR, Metcalfe CD, March RE. *International Journal of Mass Spectrometry* **2001**, *210/211*, 371-385.
(3) Justesen U. *Journal of Mass Spectrometry* **2001**, *36*, 169-178.
(4) Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
(5) Wu W, Yan CY, Li L, Liu ZQ, Liu SY. *Journal of Chromatography A* **2004**, *1047*, 213-220.
(6) Wolfender JL, Waridel P, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Analusis* **2000**, *28*, 895-906.
(7) Kuhn F, Oehme M, Romero F, Abou-Mansour E, Tabacchi R. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.
(8) Hvattum E, Ekeberg D. *Journal of Mass Spectrometry* **2003**, *38*, 43-49.
(9) Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2005**, *40*, 364-372.
(10) March RE, Miao XS, Metcalfe CD, Stobiecki M, Marczak L. *International Journal of Mass Spectrometry* **2004**, *232*, 171-183.
(11) Petsalo A, Jalonen J, Tolonen A. *Journal of Chromatography A* **2006**, *1112*, 224-231.
(12) Ablajan K, Abliz Z, Shang XY, He JM, Zhang RP, Shi JG. *Journal of Mass Spectrometry* **2006**, *41*, 352-360.
(13) Ek S, Kartimo H, Mattila S, Tolonen A. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 9834-9842.
(14) Kerhoas L, Aouak D, Cingoz A, Routaboul JM, Lepiniec L, Einhorn J, Birlirakis N. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 6603-6612.
(15) Wojcinska M, Williams J, Mabry T, Ahmed AA, Davis BD, Toth G, El-Sayed NH, Matlawska I, Clevinger J. *Natural Product Communications* **2006**, *1*, 941-948.

(16) Cuyckens F, Rozenberg R, de Hoffmann E, Claeys M. *Journal of Mass Spectrometry* **2001**, *36*, 1203-1210.
(17) Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
(18) Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
(19) Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
(20) Ferreres F, Llorach R, Gil-Izquierdo A. *Journal of Mass Spectrometry* **2004**, *39*, 312-321.
(21) Brodbelt JS, Kenttamaa HI, Cooks RG. *Organic Mass Spectrometry* **1988**, *23*, 6-9.
(22) Hvattum E, Stenstrom Y, Ekeberg D. *Journal of Mass Spectrometry* **2004**, *39*, 1570-1581.

Chapter 9: Conclusions

This dissertation represents a body of work that aims toward and succeeds in expanding the utility of mass spectrometry to characterize monoglycosyl flavonoids and flavonoid metabolites. The simplicity and the utility of the method have been proven in numerous practical applications, as described in earlier chapters. As with any analytical technique, it is important to understand the capabilities, requirements and limitations of the method.

The capabilities of the described methods should be sufficiently clear given the number of applications presented. Specifically, metal complexation combined with tandem mass spectrometry provides a means to identify flavonoids, flavonoid glycosides and flavonoid glucuronides without resorting to more difficult techniques that require larger amounts of sample or authenticated standards. Conjugated flavonoids are challenging compounds to fully identify because of many possible positions of conjugation. The glycosyl portions of the molecules are often isomeric (such as glucose vs. galactose), and these structural features must also be determined to fully identify the analyte. Mass spectrometry is a powerful analytical technique because mass is a highly specific chemical property. But mass spectrometry may be less useful in applications involving several analytes of the same or similar masses. It is not uncommon to find reports in the scientific literature of new mass spectrometric methods for resolving a

particular pair or set of isomers, such as the amino acids leucine and isoleucine. But there is so much variability in flavonoid structure that the ability to resolve specific isomers is not especially useful. Thus the most important applications of the work described here are based not on the ability to differentiate specific isomers, but rather in the discovery of systematic indicators of particular structural features such that newly-encountered compounds may be characterized and identified with very little prior information necessary. Methods were presented for determining the five most common glycosylation sites of monoglycosyl flavonoids (Chapter 3); differentiating isomeric sugars, particularly glucose and galactose, at the 3 position (Chapter 4); and determining glucuronidation sites of flavonoid metabolites (Chapter 6). Each of these methods has been used to confidently identify molecules in food or biological samples, even in cases where standards were not available for comparative purposes. Some of these molecules have not been identified previously by any other method.

The instrumentation required to perform these analyses is not particularly difficult to obtain. First, a quadrupole ion trap mass spectrometer is required. Results have only been obtained on QIT mass spectrometers, and it is believed that this type of mass spectrometer is necessary to reproduce the data presented here. The specific features that make the QIT a necessary tool are its low-energy CID processes and the availability of multiple-stage CID (MS^n). Many other

types of mass spectrometers implement CID with higher collision energies, so the results of such experiments are not directly comparable to those obtained on a QIT. More importantly, MS^n capabilities were absolutely essential to the success of the applications presented in this work. Not only is it required for differentiating glucosides from galactosides (as described in Section 4.3.2), but it is also necessary to identify the aglycon portion of flavonoid conjugates. Targeted MS^n is easily implemented on QIT mass spectrometers and comes as a standard feature on commercial instruments, whereas MS^n is difficult or impossible in most other types of mass spectrometers. The requirement of a QIT is not especially prohibitive. Mass spectrometry continues to grow in popularity, and it can be found in many laboratories that deal extensively in the molecular sciences. The QIT is one of the most cost-effective and robust types of mass spectrometers. Commercial QITs are sold by several instrument manufacturers, so availability of this instrument is not a problem.

The second instrumentation requirement is a gentle ionization source. To date, the only ionization method that had been tested on the flavonoid/metal complexes described in this work is electrospray ionization. ESI is one of the most widely-used ionization techniques for mass spectrometry and comes as a standard feature on many commercial instruments. The important characteristic of ESI that makes it especially applicable to the current work is its ability to transfer analytes to the gas phase without depositing large amounts of energy that

could disrupt fragile non-covalent species such as flavonoid/metal complexes. In fact, an attempt to analyze flavonoid/metal complexes using an ESI source known to be somewhat harsher than usual failed. It may be possible that other soft ionization methods can be used to reproduce the results shown in this work.

A third piece of instrumentation, less required than highly desirable, is an HPLC system that can be interfaced to the QIT mass spectrometer. While all of the methods described can be implemented without chromatography, this would require isolation and purification of each flavonoid analyte. It is far more efficient to forgo these steps and identify compounds in mixtures using HPLC with post-column complexation strategies. The ease and speed of this approach make it far less work-intensive than standard NMR methods.

Finally, a frank assessment of the limitations of the techniques presented here is warranted. One must recall that only flavonoids of a few specific classes (flavonols, flavones and flavanones) can be analyzed by these methods. However, flavonols and flavones occur widely throughout the plant kingdom and are thus present in nearly all plant-based food products. Flavanones occur less widely, but are especially abundant in citrus fruits, which are important commercial crops and contribute a significant portion of the flavonoids consumed in the human diet. Hence while not all flavonoids can be characterized using the described methodology, those that can are among the most widespread and important members in terms of botanical and nutritional significance.

Many flavonoids researchers are interested in quantification, not just identification. The metal complexation methods described here are not well-suited for meeting this goal. First, the formation of metal complexes involves a loss of sensitivity in the mass spectrometer of approximately two orders of magnitude. Second, it would be difficult to obtain a standard curve that is linear over a wide range of concentrations. When flavonoids and metal salts are mixed together, complexes of several different stoichiometries are formed. This was pointed out in Section 3.3.1 and Section 4.3.1, but is true for all of the metal complexation work in this dissertation. Varying the proportions of the flavonoids and the metal salts will shift the equilibrium ratios of the various complexes. Thus attempts to quantify flavonoid derivatives using metal complexation are not likely to be successful. Quantification of uncomplexed flavonoid derivatives using mass spectrometry or LC-MS is a simpler and more sensitive approach.

Another limitation lies in the fact that all identifications are based on analogy. The metal complexation strategies are developed using a small training set of flavonoids containing particular structural features of interest. Thus any compounds that contain unusual structural features (such as, for example, 8-O-glycosylation) cannot yet be identified via this methodology. At best, an incomprehensible fragmentation pattern will be obtained, signaling a breakdown in the method. At worst, the fragmentation pattern will mimic that of another structural feature, and an incorrect identification will be made. The potential for

this occurrence was realized in the case of galangin 5-O-glucuronide, recounted in Section 6.3.5. The training set of flavonoid glucuronides did not contain any 5-O-glucuronides, so it could not be anticipated that the relevant [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ complexes of flavonoid 5-O-glucuronides would fragment identically to the analogous flavonoid 3-O-glucuronide complexes. The confidence of identification is therefore proportional to the size and diversity of the training set of flavonoids used to develop the method. Additional confidence is gained as each method is successfully applied to new analytes. For example, the manganese compexation method for differentiating flavonoid glucosides and galactosides was developed using a set of only two pairs of isomers (Section 4.3.2), but the subsequent identification of two more pairs of similar isomers using the same method (Section 5.3.4) lends credence to the efficacy and wider applicability of this type of metal complex.

It is important to note that while some types of differentiation are still not possible, this is more a result of lacking sufficiently diverse molecules to create training sets than of failing to find metal complexes that systematically differentiate isomers. It is significant that thus far a successful metal complexation mode has been found for every type of flavonoid isomer differentiation that has been attempted. One may speculate that nearly any systematic structural determination of flavonoid derivatives can be made in this fashion, provided one has access to a training set of flavonoids containing the

structural features in question and a variety of metal ions and auxiliary ligands. A particular discovery made this speculation more probable. The metal complexes that provide five-way differentiation of glycosylation site always have the most trouble differentiating glycosylation at the 3-O and 4′-O positions because the diagnostic fragment ions that distinguish these two structural features are of very low abundance. However, after the discovery of the [Co(II) (FG-H) (4,7-dpphen)$_2$]$^+$ complexes for applications involving flavonoid glucuronides, the same complexes were tested on the monoglycosyl flavonoids. As recounted in Section 3.3.7, the same complex provides a robust way of determining 3-O-glycosylation. All of the complexes involving flavonoid 3-O-glucosides, 3-O-galactosides, 3-O-rhamnosides, 3-O-xylosides and 3-O-arabinofuranosides yielded a prominent fragment ion that is completely lacking from complexes of flavonoid 7-O-glycosides and 4′-O-glycosides. This method may be employed if ever there is ambiguity between 3-O-glycosylation and 4′-O-glycosylation based on results from a different metal complex. This research may then be thought of as a work in progress, as more metal complexes may be discovered in the future that will identify compounds that are currently indistinguishable.

The impact of this work has already been significant. The *Sliphium albiflorum* project (described in Section 5.3.4) provided a direct contrast between the metal complexation / tandem mass spectrometry methods used to identify the unknowns in an impure fraction and the traditional NMR methods used to identify

the remaining compounds. Jeffrey Williams of the University of Texas at Austin Department of Biology explained that 1 kg of leaves had to be harvested in order to isolate enough of the flavonoid glycosides for NMR identification. He said, only half-joking, that he was afraid of eradicating the species in his attempt to characterize it. This is a particular concern as the habitat of *Silphium albiflorum* is limited to central and north-central Texas. The isolation and purification steps required for NMR were difficult, tedious, and in the end, not completely successful. The fraction that remained impure gave ambiguous NMR results. In contrast, mass spectrometry made quick work of the sample. A small scraping of the powdered fraction, approximately 0.1 mg, was all that was needed for the analysis. Three of the four components were quickly identified, while the fourth, being less abundant in the sample, required slightly more material for a confident identification. The chromatography did not need to be highly optimized, and in fact two of the compounds co-eluted, as shown in Figure 5.8. The savings in time and effort afforded by the use of mass spectrometry instead of NMR was immediately obvious to all involved.

The ability to characterize flavonoid glucuronides may be of even greater significance. It is amazing how few *in vivo* or *in vitro* metabolism studies manage to provide complete identification of the metabolic products, despite great interest in obtaining this information. This is a testament to the difficulties involved in using established methods to provide such information. Already the metal

complexation technique has identified metabolites such as naringenin 4′-O-glucuronide and hesperetin 3′-O-glucuronide that to knowledge have not previously been identified in human biological samples. The broad applicability of the technique was demonstrated by the identification of seventeen products of an enzymatic incubation of an enzyme involved in flavonoid metabolism (Chapter 7). The elucidation of enzyme reactivity represents a significance step towards better understanding the metabolism and hence the bioactivity of dietary flavonoids. It is hoped that future researchers will continue this important work and take advantage of these new methods to obtain vital structural information from flavonoid metabolites.

References

Abe K, Katayama H, Suzuki A, Yumioka E. *Japanese Journal of Pharmacognosy-Shoyakugaku Zasshi* **1993**, *47*, 402-407.

Ablajan K, Abliz Z, Shang XY, He JM, Zhang RP, Shi JG. *Journal of Mass Spectrometry* **2006**, *41*, 352-360.

Agrawal PK, Markham KR. In *Carbon-13 NMR of Flavonoids*; Elsevier: Amsterdam, **1989**, 1-40.

Alvarez EJ, Vartanian VH, Brodbelt JS. *Analytical Chemistry* **1997**, *69*, 1147-1155.

Alvarez EJ, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 463-472.

Arts ICW, Hollman PCH, Feskens EJM, de Mesquita HBB, Kromhout D. *American Journal of Clinical Nutrition* **2001**, *74*, 227-232.

Arts ICW, Jacobs DR, Harnack LJ, Gross M, Folsom AR. *Epidemiology* **2001**, *12*, 668-675.

Arts ICW, Sesink ALA, Hollman PCH. *Free Radical Research* **2002**, *36*, 93-94.

Arts ICW, Sesink ALA, Faassen-Peters M, Hollman PCH. *British Journal of Nutrition* **2004**, *91*, 841-847.

Arts ICW, Hollman PCH. *American Journal of Clinical Nutrition* **2005**, *81*, 317s-325s.

Asam MR, Glish GL. *Journal of the American Society for Mass Spectrometry* **1997**, *8*, 987-995.

Aziz AA, Edwards CA, Lean MEJ, Crozier A. *Free Radical Research* **1998**, *29*, 257-269.

Bai Y, Song FR, Chen ML, Xing JP, Liu ZQ, Liu SY. *Analytical Sciences* **2004**, *20*, 1147-1151.

Bakhtiar A, Gleye J, Moulis C, Fouraste I. *Phytochemical Analysis* **1994**, *5*, 86-89.

Basso E, Chilin A, Guiotto A, Traldi P. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 2781-2787.

Becchi M, Fraisse D. *Biomedical and Environmental Mass Spectrometry* **1989**, *18*, 122-130.

Beck JL, Colgrave ML, Ralph SF, Sheil MM. *Mass Spectrometry Reviews* **2001**, *20*, 61-87.

Berardini N, Fezer R, Conrad J, Beifuss U, Carle R, Schieber A. *Journal of Agricultural and Food Chemistry* **2005**, *53*, 1563-1570.

Bier ME, Schwartz JC. In *Electrospray Ionization Mass Spectrometry*; Wiley: New York, **1997**, 235-289.

Blades AT, Jayaweera P, Ikonomou MG, Kebarle P. *International Journal of Mass Spectrometry and Ion Processes* **1990**, *102*, 251-267.

Boersma MG, van der Woude H, Bogaards J, Boeren S, Vervoort J, Cnubben NHP, van Iersel MLPS, van Bladeren PJ, Rietjens IMCM. *Chemical Research in Toxicology* **2002**, *15*, 662-670.
Borges C, Martinho P, Martins A, Rauter AP, Ferreira MAA. *Rapid Communications in Mass Spectrometry* **2001**, *15*, 1760-1767.
Boue SM, Carter-Wientjes CH, Shih BY, Cleveland TE. *Journal of Chromatography A* **2003**, *991*, 61-68.
Boyle SP, Dobson VL, Duthie SJ, Kyle JAM, Collins AR. *European Journal of Nutrition* **2000**, *39*, 213-223.
Bravo A, Anacona JR. *Transition Metal Chemistry* **2001**, *26*, 20-23.
Bravo L. *Nutrition Reviews* **1998**, *56*, 317-333.
Brodbelt JS, Kenttamaa HI, Cooks RG. *Organic Mass Spectrometry* **1988**, *23*, 6-9.
Brodbelt JS. *International Journal of Mass Spectrometry* **2000**, *200*, 57-69.
Brown JE, Khodr H, Hider RC, Rice-Evans CA. *Biochemical Journal* **1998**, *330*, 1173-1178.
Careri M, Elviri L, Mangia A. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 2399-2405.
Carlesso V, Fournier F, Tabet JC. *European Journal of Mass Spectrometry* **2000**, *6*, 421-428.
Chaparro AL, Vachet RW. *Journal of Mass Spectrometry* **2003**, *38*, 333-342.
Cheguillaume G, Buchmann W, Desmazieres B, Tortajada J. *Journal of Mass Spectrometry* **2004**, *39*, 368-377.
Chen H, Zuo YG, Deng YW. *Journal of Chromatography A* **2001**, *913*, 387-395.
Chen KH, Weng MS, Lin JK. *Biochemical Pharmacology* **2007**, *73*, 215-227.
Cheng ZQ, Radominska-Pandya A, Tephly TR. *Drug Metabolism and Disposition* **1999**, *27*, 1165-1170.
Chun OK, Chung SJ, Song WO. *Journal of Nutrition* **2007**, *137*, 1244-1252.
Clevinger JA, Panero JL. *American Journal of Botany* **2000**, *87*, 565-572.
Clowers BH, Hill HH. *Journal of Mass Spectrometry* **2006**, *41*, 339-351.
Conquer JA, Maiani G, Azzini E, Raguzzini A, Holub BJ. *Journal of Nutrition* **1998**, *128*, 593-597.
Constant HL, Slowing K, Graham JG, Pezzuto JM, Cordell GA, Beecher CWW. *Phytochemical Analysis* **1997**, *8*, 176-180.
Cotton FA, Wilkinson G. *Advanced inorganic chemistry: a comprehensive text*; Interscience Publishers: New York, **1966**.
Cuyckens F, Ma YL, Pocsfalvi G, Claeys M. *Analusis* **2000**, *28*, 888-895.
Cuyckens F, Rozenberg R, de Hoffmann E, Claeys M. *Journal of Mass Spectrometry* **2001**, *36*, 1203-1210.
Cuyckens F, Claeys M. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 2341-2348.

Cuyckens F, Shahat AA, Pieters L, Claeys M. *Journal of Mass Spectrometry* **2002**, *37*, 1272-1279.
Cuyckens F, Shahat AA, Van den Heuvel H, Abdel-Shafeek KA, El-Messiry MM, Seif-El Nasr MM, Pieters L, Vlietinck AJ, Claeys M. *European Journal of Mass Spectrometry* **2003**, *9*, 409-420.
Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
Cuyckens F, Claeys M. *Journal of Mass Spectrometry* **2005**, *40*, 364-372.
Davis BD, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 1287-1299.
Davis BD, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1883-1890.
Davis BD, Needs PW, Kroon PA, Brodbelt JS. *Journal of Mass Spectrometry* **2006**, *41*, 911-920.
Davis BD, Kroon PA, Brodbelt JS. *In vivo* Human Metabolites of Flavonols and Flavanones. *55th ASMS Conference on Mass Spectrometry and Allied Topics*, Indianapolis, **2007**.
Day AJ, DuPont MS, Ridley S, Rhodes M, Rhodes MJC, Morgan MRA, Williamson G. *FEBS Letters* **1998**, *436*, 71-75.
Day AJ, Williamson G. In *Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology*; Kluwer Academic / Plenum Publishers: New York, **1999**, 415-434.
Day AJ, Bao YP, Morgan MRA, Williamson G. *Free Radical Biology and Medicine* **2000**, *29*, 1234-1243.
Day AJ, Mellon F, Barron D, Sarrazin G, Morgan MRA, Williamson G. *Free Radical Research* **2001**, *35*, 941-952.
Day AJ, Williamson G. *British Journal of Nutrition* **2001**, *86*, S105-S110.
Day AJ, Gee JM, DuPont MS, Johnson IT, Williamson G. *Biochemical Pharmacology* **2003**, *65*, 1199-1206.
de Kleijn MJJ, van der Schouw YT, Wilson PWF, Adlercreutz H, Mazur W, Grobbee DE, Jacques PF. *Journal of Nutrition* **2001**, *131*, 1826-1832.
De Pascual-Teresa S, Rivas-Gonzalo JC. In *Methods in Polyphenol Analysis*; The Royal Society of Chemistry: Cambridge, UK, **2003**, 48-62.
de Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2003**, *984*, 45-58.
de Rijke E, Out P, Niessen WMA, Ariese F, Gooijer C, Brinkman UAT. *Journal of Chromatography A* **2006**, *1112*, 31-63.
de Souza RFV, Sussuchi EM, De Giovani WF. *Synthesis and Reactivity in Inorganic and Metal-Organic Chemistry* **2003**, *33*, 1125-1144.
de Souza RFV, De Giovani WF. *Spectrochimica Acta Part A* **2005**, *61*, 1985-1990.
Deng HT, Van Berkel GJ. *Journal of Mass Spectrometry* **1998**, *33*, 1080-1087.

Desaire H, Leary JA. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 1086-1094.
Desaire H, Beyer MK, Leary JA. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 517-527.
Desaire H, Leary JA. *International Journal of Mass Spectrometry* **2001**, *209*, 171-184.
Dick AJ, Redden PR, Demarco AC, Lidster PD, Grindley TB. *Journal of Agricultural and Food Chemistry* **1987**, *35*, 529-531.
Domon B, Costello CE. *Glycoconjugate Journal* **1988**, *5*, 397-409.
Dugo P, Lo Presti M, Ohman M, Fazio A, Dugo G, Mondello L. *Journal of Separation Science* **2005**, *28*, 1149-1156.
Durgo K, Vukovic L, Rusak G, Osmak M, Colic JF. *Food Technology and Biotechnology* **2007**, *45*, 69-79.
Ebner T, Burchell B. *Drug Metabolism and Disposition* **1993**, *21*, 50-55.
Edenharder R, Keller G, Platt KL, Unger KK. *Journal of Agricultural and Food Chemistry* **2001**, *49*, 2767-2773.
Ek S, Kartimo H, Mattila S, Tolonen A. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 9834-9842.
Elipe MVS. *Analytica Chimica Acta* **2003**, *497*, 1-25.
Fabre N, Rustan I, de Hoffmann E, Quetin-Leclercq J. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
Fang J, Zhou Q, Liu LZ, Xia C, Hu X, Shi X, Jiang BH. *Carcinogenesis* **2007**, *28*, 858-864.
Fernandez MT, Mira ML, Florencio MH, Jennings KR. *Journal of Inorganic Biochemistry* **2002**, *92*, 105-111.
Ferreres F, Gil MI, Tomas-Barberan FA. *Food Research International* **1996**, *29*, 389-395.
Ferreres F, Silva BM, Andrade PB, Seabra RM, Ferreira MA. *Phytochemical Analysis* **2003**, *14*, 352-359.
Ferreres F, Llorach R, Gil-Izquierdo A. *Journal of Mass Spectrometry* **2004**, *39*, 312-321.
Franski R, Bednarek P, Wojtaszek P, Stobiecki M. *Journal of Mass Spectrometry* **1999**, *34*, 486-495.
Franski R, Eitner K, Sikorska M, Matlawska I, Stobiecki M. *Phytochemical Analysis* **2003**, *14*, 170-175.
Frison-Norrie S, Sporns P. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 2782-2787.
Gabelica V, De Pauw E. *Mass Spectrometry Reviews* **2005**, *24*, 566-587.
Gatlin CL, Turecek F, Vaisar T. *Analytical Chemistry* **1994**, *66*, 3950-3958.
Gaucher SP, Leary JA. *Analytical Chemistry* **1998**, *70*, 3009-3014.

Gaucher SP, Leary JA. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 269-272.
Gaucher SP, Leary JA. *International Journal of Mass Spectrometry* **2000**, *197*, 139-148.
Gee JM, DuPont MS, Day AJ, Plumb GW, Williamson G, Johnson IT. *Journal of Nutrition* **2000**, *130*, 2765-2771.
Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM. *American Journal of Clinical Nutrition* **2002**, *75*, 880-886.
Gennaro L, Leonardi C, Esposito F, Salucci M, Maiani G, Quaglia G, Fogliano V. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 1904-1910.
Gester S, Metz P, Zierau O, Vollmer G. *Tetrahedron* **2001**, *57*, 1015-1018.
Graf BA, Milbury PE, Blumberg JB. *Journal of Medicinal Food* **2005**, *8*, 281-290.
Grayer RJ, Kite GC, Abou-Zaid M, Archer LJ. *Phytochemical Analysis* **2000**, *11*, 257-267.
Grayer RJ, Kite GC, Veitch NC, Eckert MR, Marin PD, Senanayake P, Paton AJ. *Biochemical Systematics and Ecology* **2002**, *30*, 327-342.
Green MD, Oturu EM, Tephly TR. *Drug Metabolism and Disposition* **1994**, *22*, 799-805.
Green MD, Tephly TR. *Drug Metabolism and Disposition* **1998**, *26*, 860-867.
Ha JH, Lee JT, Cho IH, Chun KA, Park GE, Choi HC, Lee KY, Kim SH, Suk K, Kim IK, Lee MG. *Phytomedicine* **2007**, *14*, 232-235.
Harborne JB, Boardley M. *Journal of Chromatography A* **1984**, *299*, 377-385.
Harborne JB. *The Flavonoids: Advances in Research Since 1986*; Chapman & Hall: London; New York, **1994**.
Harborne JB, Baxter H. *The Handbook of Natural Flavonoids, vol. 1 & 2*; John Wiley & Sons: Chichester, **1999**.
Hayen H, Alvarez-Grima MM, Debnath SC, Noordermeer JWM, Karst U. *Analytical Chemistry* **2004**, *76*, 1063-1068.
Heim KE, Tagliaferro AR, Bobilya DJ. *Journal of Nutritional Biochemistry* **2002**, *13*, 572-584.
Henderson W, Evans C. *Inorganica Chimica Acta* **1999**, *294*, 183-192.
Hertog MGL, Feskens EJM, Hollman PCH, Katan MB, Kromhout D. *Lancet* **1993**, *342*, 1007-1011.
Hertog MGL, Kromhout D, Aravanis C, Blackburn H, Buzina R, Fidanza F, Giampaoli S, Jansen A, Menotti A, Nedeljkovic S, Pekkarinen M, Simic BS, Toshima H, Feskens EJM, Hollman PCH, Katan MB. *Archives of Internal Medicine* **1995**, *155*, 381-386.
Hertog MGL, Feskens EJM, Kromhout D. *Lancet* **1997**, *349*, 699-699.
Hertog MGL, Sweetman PM, Fehily AM, Elwood PC, Kromhout D. *American Journal of Clinical Nutrition* **1997**, *65*, 1489-1494.

Himeji M, Ohtsuki T, Fukazawa H, Tanaka M, Yazaki S, Ui S, Nishio K, Yamamoto H, Tasaka K, Mimura A. *Cancer Letters* **2007**, *245*, 269-274.
Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. *Stroke* **2000**, *31*, 2301-2306.
Hirvonen T, Pietinen P, Virtanen M, Ovaskainen ML, Hakkinen S, Albanes D, Virtamo J. *Epidemiology* **2001**, *12*, 62-67.
Hirvonen T, Virtamo J, Korhonen P, Albanes D, Pietinen P. *Cancer Causes & Control* **2001**, *12*, 789-796.
Ho YP, Huang PC, Deng KH. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 114-121.
Hollman PCH, vanTrijp JMP, Buysman MNCP. *Analytical Chemistry* **1996**, *68*, 3511-3515.
Hong YJ, Mitchell AE. *Chemical Research in Toxicology* **2006**, *19*, 1525-1532.
Huck CW, Buchmeiser MR, Bonn GK. *Journal of Chromatography A* **2002**, *943*, 33-38.
Hughes RJ, Croley TR, Metcalfe CD, March RE. *International Journal of Mass Spectrometry* **2001**, *210/211*, 371-385.
Hvattum E. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 655-662.
Hvattum E, Ekeberg D. *Journal of Mass Spectrometry* **2003**, *38*, 43-49.
Hvattum E, Stenstrom Y, Ekeberg D. *Journal of Mass Spectrometry* **2004**, *39*, 1570-1581.
Jellen EE, Chappell AM, Ryzhov V. *Rapid Communications in Mass Spectrometry* **2002**, *16*, 1799-1804.
Ju W, Wang X, H. S, Chen W, Belinsky SA, Lin Y. *Molecular Pharmacology* **2007**, *71*, 1381-1388.
Justesen U, Knuthsen P, Leth T. *Journal of Chromatography A* **1998**, *799*, 101-110.
Justesen U. *Journal of Chromatography A* **2000**, *902*, 369-379.
Justesen U. *Journal of Mass Spectrometry* **2001**, *36*, 169-178.
Justesen U, Knuthsen P. *Food Chemistry* **2001**, *73*, 245-250.
Kanadaswami C, Lee LT, Lee PPH, Hwang JJ, Ke FC, Huang YT, Lee MT. *In Vivo* **2005**, *19*, 895-909.
Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. *Bioscience Biotechnology and Biochemistry* **1999**, *63*, 896-899.
Kazazic SP, Butkovic V, Srzic D, Klasinc L. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 8391-8396.
Keki S, Deak G, Zsuga M. *Journal of Mass Spectrometry* **2001**, *36*, 1312-1316.
Keli SO, Hertog MGL, Feskens EJM, Kromhout D. *Archives of Internal Medicine* **1996**, *156*, 637-642.
Kerhoas L, Aouak D, Cingoz A, Routaboul JM, Lepiniec L, Einhorn J, Birlirakis N. *Journal of Agricultural and Food Chemistry* **2006**, *54*, 6603-6612.

King CD, Green MD, Rios GR, Coffman BL, Owens IS, Bishop WP, Tephly TR. *Archives of Biochemistry and Biophysics* **1996**, *332*, 92-100.
King CD, Rios GR, Green MD, Tephly TR. *Current Drug Metabolism* **2000**, *1*, 143-161.
Knekt P, Jarvinen R, Reunanen A, Maatela J. *British Medical Journal* **1996**, *312*, 478-481.
Knekt P, Jarvinen R, Seppanen R, Heliovaara M, Teppo L, Pukkala E, Aromaa A. *American Journal of Epidemiology* **1997**, *146*, 223-230.
Knekt P, Kumpulainen J, Jarvinen R, Rissanen H, Heliovaara M, Reunanen A, Hakulinen T, Aromaa A. *American Journal of Clinical Nutrition* **2002**, *76*, 560-568.
Konig S, Leary JA. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1125-1134.
Kris-Etherton PM, Hecker KD, Bonanome A, Coval SM, Binkoski AE, Hilpert KF, Griel AE, Etherton TD. *American Journal of Medicine* **2002**, *113*, 71-88.
Kroon PA, Clifford MN, Crozier A, Day AJ, Donovan JL, Manach C, Williamson G. *American Journal of Clinical Nutrition* **2004**, *80*, 15-21.
Kuhn F, Oehme M, Romero F, Abou-Mansour E, Tabacchi R. *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.
Kuhnau J. *World Review of Nutrition and Dietetics* **1976**, *24*, 117.
Kumar MAS, Nair M, Hema PS, Mohan J, Santhoshkumar TR. *Molecular Carcinogenesis* **2007**, *46*, 231-241.
Kyle J, Duthie G. *Natural Product Communications* **2006**, *1*, 1049-1060.
Lavanant H, Virelizier H, Hoppilliard Y. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1217-1221.
Lee JH, Lee JY, Kim KN, Kim HS. *Food Science and Biotechnology* **2003**, *12*, 415-418.
Lentini A, Forni C, Provenzano B, Beninati S. *Amino Acids* **2007**, *32*, 95-100.
Li QM, Van den Heuvel H, Dillen L, Claeys M. *Biological Mass Spectrometry* **1992**, *21*, 213-221.
Li QM, Claeys M. *Biological Mass Spectrometry* **1994**, *23*, 406-416.
Lin LZ, Harnly JM. *Journal of Agricultural and Food Chemistry* **2007**, *55*, 1084-1096.
Lin YY, Ng KJ, Yang SJ. *Journal of Chromatography A* **1993**, *629*, 389-393.
Llorach R, Gil-Izquierdo A, Ferreres F, Tomas-Barberan FA. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 3895-3899.
Lommen A, Godejohann M, Venema DP, Hollman PCH, Spraul M. *Analytical Chemistry* **2000**, *72*, 1793-1797.
Lopez LL, Tiller PR, Senko MW, Schwartz JC. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 663-668.

Ma G, Yang C, Qu Y, Wei H, Zhang T, Zhang N. *Chemico-Biological Interactions* **2007**, *167*, 153-160.
Ma YL, Li QM, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
Ma YL, Van den Heuvel H, Claeys M. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 1932-1942.
Ma YL, Vedernikova I, Van den Heuvel H, Claeys M. *Journal of the American Society for Mass Spectrometry* **2000**, *11*, 136-144.
Ma YL, Cuyckens F, Van den Heuvel H, Claeys M. *Phytochemical Analysis* **2001**, *12*, 159-165.
Mabry TJ, Markham KR, Thomas MB. *The Systematic Identification of Flavonoids*; Springer-Verlag: New York, **1970**.
Mabry TJ, Ulubelen A. In *Biochemical Applications of Mass Spectrometry, 1st Supplemental Volume*; Wiley: New York, **1980**, 1131-1158.
Maillard MN, Giampaoli P, Cuvelier ME. *Talanta* **1996**, *43*, 339-347.
Manach C, Morand C, Gil-Izquierdo A, Bouteloup-Demange C, Remesy C. *European Journal of Clinical Nutrition* **2003**, *57*, 235-242.
Manach C, Donovan JL. *Free Radical Research* **2004**, *38*, 771-785.
Manthey JA, Guthrie N, Grohmann K. *Current Medicinal Chemistry* **2001**, *8*, 135-153.
Manthey JA, Guthrie N. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 5837-5843.
March RE, Miao XS. *International Journal of Mass Spectrometry* **2004**, *231*, 157-167.
March RE, Miao XS, Metcalfe CD, Stobiecki M, Marczak L. *International Journal of Mass Spectrometry* **2004**, *232*, 171-183.
Markham KR. *Techniques of Flavonoid Identification*; Academic Press: London; New York, **1982**.
Marotti M, Piccaglia R. *Journal of Food Science* **2002**, *67*, 1229-1232.
Martins HFP, Leal JP, Fernandez MT, Lopes VHC, Cordeiro MNDS. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 848-861.
Matsumoto H, Ikoma Y, Sugiura M, Yano M, Hasegawa Y. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 6653-6659.
Mauri PL, Iemoli L, Gardana C, Riso P, Simonetti P, Porrini M, Pietta PG. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 924-931.
McClellan JE, Murphy JP, Mulholland JJ, Yost RA. *Analytical Chemistry* **2002**, *74*, 402-412.
Mennen LI, Sapinho D, de Bree A, Arnault N, Bertrais S, Galan P, Hercberg S. *Journal of Nutrition* **2004**, *134*, 923-926.
Middleton E, Kandaswami C, Theoharides TC. *Pharmacological Reviews* **2000**, *52*, 673-751.

Moridani MY, Pourahmad J, Bui H, Siraki A, O'Brien PJ. *Free Radical Biology and Medicine* **2003**, *34*, 243-253.
Morley KL, Ferguson PJ, Koropatnick J. *Cancer Letters* **2007**, *251*, 168-178.
Mullen W, Graf BA, Caldwell ST, Hartley RC, Duthie GG, Edwards CA, Lean MEJ, Crozier A. *Journal of Agricultural and Food Chemistry* **2002**, *50*, 6902-6909.
Mullen W, Boitier A, Stewart AJ, Crozier A. *Journal of Chromatography A* **2004**, *1058*, 163-168.
Mullen W, Edwards CA, Crozier A. *British Journal of Nutrition* **2006**, *96*, 107-116.
Murota K, Mitsukuni Y, Ichikawa M, Tsushida T, Miyamoto S, Terao J. *Journal of Agricultural and Food Chemistry* **2004**, *52*, 1907-1912.
Needs PW, Williamson G. *Carbohydrate Research* **2001**, *330*, 511-515.
Needs PW, Kroon PA. *Tetrahedron* **2006**, *62*, 6862-6868.
Neuhouser ML. *Nutrition and Cancer-an International Journal* **2004**, *50*, 1-7.
Nielsen SE, Freese R, Cornett C, Dragsted LO. *Analytical Chemistry* **2000**, *72*, 1503-1509.
Niessen WMA. *Liquid Chromatography-Mass Spectrometry*; Marcel Dekker: New York, **1999**.
Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. *American Journal of Clinical Nutrition* **2001**, *74*, 418-425.
Nindi MM, Kgarebe BV, Wolfender JL, Abegaz BM. *Phytochemical Analysis* **1999**, *10*, 69-75.
Ninfali P, Bacchiocca M, Antonelli A, Biagiotti E, Di Gioacchino AM, Piccoli G, Stocchi V, Brandi G. *Phytomedicine* **2007**, *14*, 216-221.
O'Leary KA, Day AJ, Needs PW, Sly WS, O'Brien NM, Williamson G. *FEBS Letters* **2001**, *503*, 103-106.
O'Leary KA, Day AJ, Needs PW, Mellon FA, O'Brien NM, Williamson G. *Biochemical Pharmacology* **2003**, *65*, 479-491.
Oleszek W, Lee CY, Jaworski AW, Price KR. *Journal of Agricultural and Food Chemistry* **1988**, *36*, 430-432.
Oleszek W. *Phytochemistry Reviews* **2002**, *1*, 163-166.
Paganga G, Rice-Evans CA. *FEBS Letters* **1997**, *401*, 78-82.
Park YK, Lee CY. *Journal of Agricultural and Food Chemistry* **1996**, *44*, 34-36.
Pastene ER, Wilkomirsky T, Bocaz G, Havel J, Peric I, Vega M, Gonzalez M, Alderete J. *Boletin De La Sociedad Chilena De Quimica* **2001**, *46*, 449-457.
Patil BS, Pike LM, Hamilton BK. *New Phytologist* **1995**, *130*, 349-355.
Patil BS, Pike LM, Yoo KS. *Journal of the American Society for Horticultural Science* **1995**, *120*, 909-913.
Petsalo A, Jalonen J, Tolonen A. *Journal of Chromatography A* **2006**, *1112*, 224-231.

Pietta P, Facino RM, Carini M, Mauri P. *Journal of Chromatography A* **1994**, *661*, 121-126.
Pikulski M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2003**, *14*, 1437-1453.
Pikulski M, Wilson JJ, Aguilar A, Brodbelt JS. *Analytical Chemistry* **2006**, *78*, 8512-8517.
Pikulski M, Aguilar A, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2007**, *18*, 422-431.
Plumb GW, O'Leary K, Day AJ, Williamson G. In *Methods in Polyphenol Analysis*; The Royal Society of Chemistry: Cambridge, UK, **2003**, 177-186.
Pramanik BN, Bartner PL, Mirza UA, Liu YH, Ganguly AK. *Journal of Mass Spectrometry* **1998**, *33*, 911-920.
Prasain JK, Patel R, Kirk M, Wilson L, Botting N, Darley-Usmar VM, Barnes S. *Journal of Mass Spectrometry* **2003**, *38*, 764-771.
Prasain JK, Wang CC, Barnes S. *Free Radical Biology and Medicine* **2004**, *37*, 1324-1350.
Proctor G, Leonard J, Lygo B. *Advanced Practical Organic Chemistry*; CRC Press: **1994**.
Proteggente AR, Basu-Modak S, Kuhnle G, Gordon MJ, Youdim K, Tyrrell R, Rice-Evans CA. *Photochemistry and Photobiology* **2003**, *78*, 256-261.
Raffaelli A, Moneti G, Mercati V, Toja E. *Journal of Chromatography A* **1997**, *777*, 223-231.
Rauha JP, Vuorela H, Kostiainen R. *Journal of Mass Spectrometry* **2001**, *36*, 1269-1280.
Rhodes MJC, Price KR. *Food Chemistry* **1996**, *57*, 113-117.
Rice-Evans C, Packer L. *Flavonoids in Health and Disease*; Marcel Dekker: New York, **1998**.
Rice-Evans CA, Miller NJ, Paganga G. *Free Radical Biology and Medicine* **1996**, *20*, 933-956.
Rimm EB, Katan MB, Ascherio A, Stampfer MJ, Willett WC. *Annals of Internal Medicine* **1996**, *125*, 384-389.
Robards K, Antolovich M. *Analyst* **1997**, *122*, 11R-34R.
Roseiro LB, Viala D, Besle JM, Carnat A, Fraisse D, Chezal JM, Lamaison JL. *International Dairy Journal* **2005**, *15*, 579-584.
Ross JA, Kasum CM. *Annual Review of Nutrition* **2002**, *22*, 19-34.
Sakagami H, Kobayashi M, Chien CH, Kanegae H, Kawase M. *In Vivo* **2007**, *21*, 311-320.
Sakushima A, Nishibe S, Brandenberger H. *Biomedical and Environmental Mass Spectrometry* **1989**, *18*, 809-815.
Sampson L, Rimm E, Hollman PCH, de Vries JHM, Katan MB. *Journal of the American Dietetic Association* **2002**, *102*, 1414-1420.

Sanchez-Rabaneda F, Jauregui O, Casals I, Andres-Lacueva C, Izquierdo-Pulido M, Lamuela-Raventos RM. *Journal of Mass Spectrometry* **2003**, *38*, 35-42.
Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Bastida J, Viladomat F, Codina C. *Journal of Chromatography A* **2003**, *1008*, 57-72.
Sanchez-Rabaneda F, Jauregui O, Lamuela-Raventos RM, Viladomat F, Bastida J, Codina C. *Rapid Communications in Mass Spectrometry* **2004**, *18*, 553-563.
Satterfield M, Brodbelt JS. *Analytical Chemistry* **2000**, *72*, 5898-5906.
Satterfield M, Black DM, Brodbelt JS. *Journal of Chromatography B* **2001**, *759*, 33-41.
Satterfield M, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 537-549.
Scalbert A, Manach C, Morand C, Remesy C, Jimenez L. *Critical Reviews in Food Science and Nutrition* **2005**, *45*, 287-306.
Schels H, Zinsmeister HD, Pfleger K. *Phytochemistry* **1977**, *16*, 1019-1023.
Schels H, Zinsmeister HD, Pfleger K. *Phytochemistry* **1978**, *17*, 523-526.
Schieber A, Keller P, Carle R. *Journal of Chromatography A* **2001**, *910*, 265-273.
Schieber A, Keller P, Streker P, Klaiber I, Carle R. *Phytochemical Analysis* **2002**, *13*, 87-94.
Schieber A, Berardini N, Carle R. *Journal of Agricultural and Food Chemistry* **2003**, *51*, 5006-5011.
Sesink ALA, O'Leary KA, Hollman PCH. *Journal of Nutrition* **2001**, *131*, 1938-1941.
Sesink ALA, Arts ICW, Faassen-Peters M, Hollman PCH. *Journal of Nutrition* **2003**, *133*, 773-776.
Sesso HD, Gaziano JM, Liu S, Buring JE. *American Journal of Clinical Nutrition* **2003**, *77*, 1400-1408.
Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2000**, *35*, 566-571.
Shen J, Brodbelt J. *Journal of the American Society for Mass Spectrometry* **1999**, *10*, 126-135.
Shen J, Brodbelt JS. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 1381-1389.
Shimoi K, Okada H, Furugori M, Goda T, Takase S, Suzuki M, Hara Y, Yamamoto H, Kinae N. *FEBS Letters* **1998**, *438*, 220-224.
Siegler DS. *Plant Secondary Metabolism, part I*; Kluwer Academic: Boston, **1998**.
Sleno L, Volmer DA. *Journal of Mass Spectrometry* **2004**, *39*, 1091-1112.
Smith G, Leary JA. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 953-957.
Soczynska-Kordala M, Bakowska A, Oszmianski J, Gabrielska J. *Cellular & Molecular Biology Letters* **2001**, *6*, 277-281.

Stafford GC, Kelley PE, Syka JEP, Reynolds WE, Todd JFJ. *International Journal of Mass Spectrometry and Ion Processes* **1984**, *60*, 85-98.
Stobiecki M, Olechnowicz-Stepien W, Rzadkowska-Bodalska H, Cisowski W, Budko E. *Biomedical and Environmental Mass Spectrometry* **1988**, *15*, 589-594.
Stobiecki M. *Phytochemistry* **2000**, *54*, 237-256.
Takahashi T, Kobori M, Shinmoto H, Tsushida T. *Bioscience Biotechnology and Biochemistry* **1998**, *62*, 2199-2204.
Takayama M, Fukai T, Hano Y, Nomura T. *Heterocycles* **1992**, *33*, 405-434.
Thomasset SC, Berry DP, Garcea G, Marczylo T, Steward WP, Gescher AJ. *International Journal of Cancer* **2007**, *120*, 451-458.
Thurman EM, Mills MS. *Solid Phase Extraction: Principles and Practice*; Wiley: New York, **1998**.
Todd JFJ. In *Practical Aspects of Ion Trap Mass Spectrometry, vol. 1*; CRC Press: Boca Raton, **1995**, 3-24.
Tokusoglu O, Unal MK, Yildirim Z. *Acta Chromatographica* **2003**, *13*, 196-207.
Tozuka Z, Kaneko H, Shiraga T, Mitani Y, Beppu M, Terashita S, Kawamura A, Kagayama A. *Journal of Mass Spectrometry* **2003**, *38*, 793-808.
Tsushida T, Suzuki M. *Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi* **1995**, *42*, 100-108.
Vaidyanathan JB, Walle T. *Drug Metabolism and Disposition* **2002**, *30*, 897-903.
Vaisar T, Gatlin CL, Turecek F. *International Journal of Mass Spectrometry and Ion Processes* **1997**, *162*, 77-87.
Vaisar T, Gatlin CL, Rao RD, Seymour JL, Turecek F. *Journal of Mass Spectrometry* **2001**, *36*, 306-316.
Vartanian VH, Goolsby B, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **1998**, *9*, 1089-1098.
Vestal ML. *Chemical Reviews* **2001**, *101*, 361-375.
Vrkic AK, O'Hair RAJ. *Journal of the American Society for Mass Spectrometry* **2004**, *15*, 715-724.
Walle T, Walle UK, Halushka PV. *Journal of Nutrition* **2001**, *131*, 2648-2652.
Waller GR, Dermer OC. *Biochemical Applications of Mass Spectrometry: First Supplementary Volume*; Wiley: New York, **1980**.
Wang J, Sporns P. *Journal of Agricultural and Food Chemistry* **2000**, *48*, 1657-1662.
Waridel P, Wolfender JL, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Journal of Chromatography A* **2001**, *926*, 29-41.
Weber G. *Chromatographia* **1988**, *26*, 133-138.
Williamson G, Day AJ, Plumb GW, Couteau D. *Biochemical Society Transactions* **2000**, *28*, 16-22.
Williamson G. *Phytochemistry Reviews* **2002**, *1*, 215-222.

Williamson G, Barron D, Shimoi K, Terao J. *Free Radical Research* **2005**, *39*, 457-469.
Wittig J, Herderich M, Graefe EU, Veit M. *Journal of Chromatography B* **2001**, *753*, 237-243.
Wojcinska M, Williams J, Mabry T, Ahmed AA, Davis BD, Toth G, El-Sayed NH, Matlawska I, Clevinger J. *Natural Product Communications* **2006**, *1*, 941-948.
Wolfender JL, Maillard M, Marston A, Hostettmann K. *Phytochemical Analysis* **1992**, *3*, 193-214.
Wolfender JL, Waridel P, Ndjoko K, Hobby KR, Major HJ, Hostettmann K. *Analusis* **2000**, *28*, 895-906.
Wolfender JL, Ndjoko K, Hostettmann K. *Journal of Chromatography A* **2003**, *1000*, 437-455.
Wolfender JL, Queiroz EF, Hostettmann K. *Magnetic Resonance in Chemistry* **2005**, *43*, 697-709.
Wu W, Yan CY, Li L, Liu ZQ, Liu SY. *Journal of Chromatography A* **2004**, *1047*, 213-220.
Xu YD, Zhang X, Yergey AL. *Journal of the American Society for Mass Spectrometry* **1996**, *7*, 25-29.
Yamashita M, Fenn JB. *Journal of Physical Chemistry* **1984**, *88*, 4451-4459.
Yeh TC, Chiang PC, Li TK, Hsu JL, Lin CJ, Wang SW, Peng CY, Guh JH. *Biochemical Pharmacology* **2007**, *73*, 782-792.
Yochum L, Kushi LH, Meyer K, Folsom AR. *American Journal of Epidemiology* **1999**, *149*, 943-949.
Youdim KA, Dobbie MS, Kuhnle G, Proteggente AR, Abbott NJ, Rice-Evans C. *Journal of Neurochemistry* **2003**, *85*, 180-192.
Zhang J, Brodbelt JS. *Journal of Mass Spectrometry* **2003**, *38*, 555-572.
Zhang J, Satterfield MB, Brodbelt JS, Britz SJ, Clevidence B, Novotny JA. *Analytical Chemistry* **2003**, *75*, 6401-6407.
Zhang J, Brodbelt J. *Journal of the American Chemical Society* **2004**, *126*, 5906-5919.
Zhang J, Brodbelt JS. *Analyst* **2004**, *129*, 1227-1233.
Zhang J, Brodbelt JS. *Journal of the American Society for Mass Spectrometry* **2005**, *16*, 139-151.
Zhang J, Brodbelt JS. *Analytical Chemistry* **2005**, *77*, 1761-1770.
Zhang J, Wang JM, Brodbelt JS. *Journal of Mass Spectrometry* **2005**, *40*, 350-363.
Zhang L, Lin G, Zuo Z. *Life Sciences* **2006**, *78*, 2772-2780.

VITA

Barry D Davis was born in Brooklyn, New York on December 22, 1976 to Barry A Davis and Norma Pestano. After graduating from Hunter College High School, New York, New York, in 1995, he enrolled at the Massachusetts Institute of Technology. He received the B. S. degree in chemistry in May 1999. Afterwards he worked as an analytical chemist at Epix Medical in Cambridge, Massachusetts. In August 2002 he began graduate studies in the Department of Chemistry and Biochemistry at the University of Texas at Austin.

Permanent Address: 929 Massachusetts Ave. Apt. 1A, Cambridge MA 02139

This dissertation was typed by the author.